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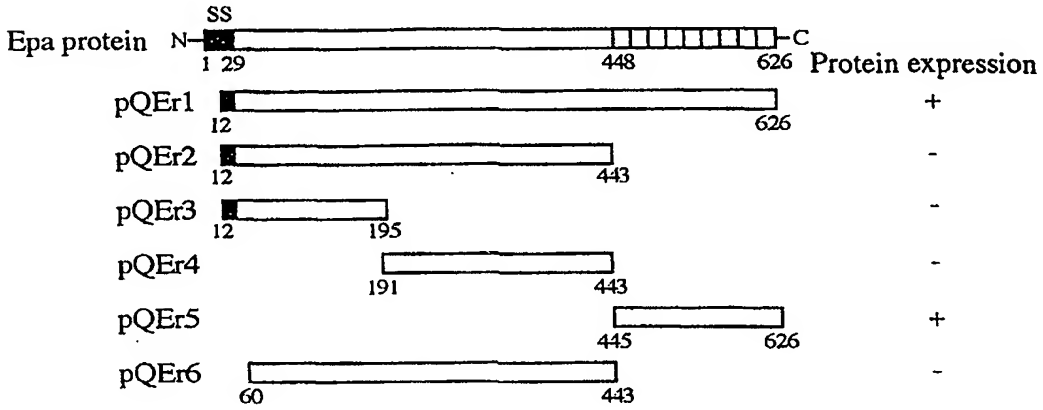

(57) Abstract

The present invention relates to the identification of a subunit vaccine to prevent or treat widespread infection of *Erysipelothrix rhusiopathiae*. In particular, the *E. rhusiopathiae* protective antigen, and more specifically, the amino terminal part of this antigen, was identified as a vaccine antigen. In a specific embodiment, a purified protein corresponding to amino acid residues 12–195 of the protective antigen protected mice and pigs from a lethal challenge with *E. rhusiopathiae*.

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ANTIGEN OF ERYSIPELOTHIRX RHUSIOPATHIAE COMPRISING AN IMMUNO-PROTECTIVE EPITOPE

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FIELD OF THE INVENTION

The present invention relates to the identification of a subunit vaccine to prevent or treat widespread infection of *Erysipelothrix rhusiopathiae*. In particular, the *E. rhusiopathiae* protective antigen, and more specifically, the amino terminal part of this antigen, was identified as a vaccine antigen.

BACKGROUND OF THE INVENTION

The gram-positive bacterium *E. rhusiopathiae* is the causative agent of erysipelas in animals and erysipeloid in humans (Wood, J. Am. Vet. Med. Assoc., 184:944-949, 1984; Wood, Diseases of Swine, Erysipelas, p. 475-486, Iowa State University Press: Ames, Iowa, 1992). The organism causes great economic losses to the swine and turkey industries (Wood, *supra*, 1992). In swine, the organism may cause acute septicemic disease or chronic disease typically characterized by endocarditis and polyarthritis (Wood, *supra*, 1992). Live attenuated vaccines or bacterins have been used for the control of swine erysipelas for many years. However, it has been suggested that the currently available vaccines do not prevent the chronic form of the disease and that vaccination may cause an increase in arthritis lesions (Wood, *supra*, 1992). Thus, there is a clear need for the development of more effective and safer vaccine.

In erysipelas, antibodies against *E. rhusiopathiae* have been known to play an important role in protection (Wood, *supra*, 1984; Wood, *supra*, 1992; and, Yokomizo and Isayama, Res. Vet. Sci., 13:294-296, 1972), suggesting that antibodies against cell surface component(s) of the organism are important in protective

immunity. However, studies on the immunogenicity of antigens in these organism have been few (Lachmann and Deicher, Infect. Immun., 52:818-822, 1986) and those antigen(s) involved in protection have not been fully characterized.

Previously, the capsule was shown to be the major virulence determinant of *E. rhusiopathiae* by protecting the organism from phagocytosis by polymorphonuclear leukocytes and intracellular killing by macrophages (Shimoji *et al.*, Infect. Immun., 62:2806-2810, 1994; Shimoji *et al.*, Infect. Immun., 64:1789-1793, 1996). However, the capsule of *E. rhusiopathiae* is poorly immunogenic and mice immunized with purified capsular antigen were not protected from subsequent lethal challenge with a virulent homologous strain, suggesting that molecule(s) on the cell surface other than capsular antigen may be important in inducing protective antibodies.

It has been reported that 200-kDa glycolipoprotein complex antigen in the culture supernatant is a protective antigen of *E. rhusiopathiae* (White and Verwey, Infect. Immun., 1:380-386, 1970; White and Verwey, Infect. Immun. 1:387-393, 1970). This antigen was able to adsorb passively protective antibodies from rabbit antiserum produced by immunization with whole bacterial culture, suggesting that this antigen is a major protective antigen of the organism. It has also been reported that a 66- to 64-kDa protein in a Triton X-100 extract of cell surface antigens is a protective molecule (Galan and Timoney, Infect. Immun., 58:3116-3121, 1990). However, mice immunized with the recombinant 66- to 64-kDa protein showed incomplete protection (Galan and Timoney, *supra*, 1990).

Recently, the cloning of a gene encoding the cell surface protein of *E. rhusiopathiae* (SpaA) was described (Makino *et al.*, Microb. Pathog., 25:101-109, 1998). In protection assays with *E. coli* modified to express SpaA antigens, an SpaA product lacking the C-terminal portion (which contained eight repeats) lost its ability to show protection, while full-length SpaA was protective. The C-terminal portion was found to be essential for binding of SpaA to bacterial cell surfaces, and the authors concluded that the C-terminal portion was a candidate for a subunit vaccine against erysipelas. In that study, however, protection experiments with the isolated protein were not conducted.

Thus, the efforts to identify a protective antigen in *E. rhusiopathiae* have been inconclusive, and it is unknown whether a single or multiple antigens are necessary to provide complete protection from infection.

SUMMARY OF THE INVENTION

The present invention advantageously identifies the immuno-protective epitope from an *Erysipelothrix rhusiopathiae* bacterium. Thus, in one aspect, a vaccine comprising an immunogenic polypeptide of *E. rhusiopathiae*, wherein the immuogenic polypeptide comprises an immuno-protective epitope which is from an N-terminal region of an erysipelas protective antigen (Epa), and an adjuvant, is provided. Moreover, the invention further provides a polypeptide comprising an amino acid sequence corresponding to about 25 residues of an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 447. In a specific embodiment, the polypeptide has a sequence of SEQ ID NO: 3. Such a polypeptide can be used to prepare a vaccine of the invention, or in an immunoassay.

The invention further provides a nucleic acid encoding a polypeptide comprising an amino acid sequence corresponding to about 25 residues of an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 447. The nucleic acid can be provided in an expression vector, *e.g.*, for a vector vaccine.

The invention further provides a method for protecting an animal from infection by *E. rhusiopathiae* comprising administering an immunologically effective amount of the vaccine of the invention, or alternatively, an immuno-protective amount of the expression vector of the invention, to the animal. An alternative method for protecting an animal from infection by *E. rhusiopathiae* involves administering an immuno-protective amount of an antibody generated against or purified with an immuno-protective epitope which is from an N-terminal region of an erysipelas protective antigen (Epa) to the animal. Preferably, the animal is a turkey or a pig. Vaccination of humans is also envisioned.

In yet another embodiment, the invention provides a method for detecting the presence of protective antibodies to *E. rhusiopathiae*. The method comprises detecting binding of antibodies from a biological sample with a polypeptide comprising an immuno-protective epitope of Epa protein of *E. rhusiopathiae*. Such

binding occurs when the biological sample is contacted with the polypeptide under conditions that permit binding of antibody in the biological sample with the polypeptide. Also provided is a kit for detecting the presence of protective antibodies to *E. rhusiopathiae*, which kit comprises a polypeptide comprising the immuno-protective epitope of Epa protein and an antibody detector.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Partial restriction map of the insert of pERc6 and its deletion derivatives. The positions of the ORFs and the incomplete ORFs are indicated by open boxes; arrows indicate the direction of transcription. Sites of restriction enzymes presented as boldface characters are located within multi-cloning-site of the vector pBK-CMV.

FIGURE 2. Nucleotide sequence (SEQ ID NO:1) of the *Epa* gene and upstream region from *E. rhusiopathiae* strain Fujisawa. The deduced amino acid sequence (SEQ ID NO:2) is indicated in single-letter code under the nucleotide sequence. Numbers on the right of each line refer to amino acid positions. The arrowhead designates the proposed site for the cleavage and removal of the signal peptide between amino acids 29 and 30. The putative promoter boxes (-35 and -10), a putative ribosomal binding site (rbs) and LPXTGX (SEQ ID NO:3) motif are indicated (double underline). The nine sequence repeats were indicated as R1 to R9 under single lines. *, stop codon. The nucleotide sequence data of the insert of pERc6 will appear in DDBJ/EMBL/GenBank database under the accession number AB017447.

FIGURE 3. Alignment of C-terminal region of *E. rhusiopathiae* Epa and that of *S. pneumoniae* PspA (SEQ ID NO:4). The predicted amino acid sequences of C-terminal repeat domain of Epa and that of PspA were aligned. Identical residues are marked with asterisks and conservative substitutions are indicated with dots. Dashes represent gaps introduced to produce optimal alignment. Numbers on the right of each line refer to amino acid positions.

FIGURE 4. Schematic representation of the expression plasmids and the expression of fusion proteins. The expression of the fusion proteins is indicated

on the right side. The signal sequence (SS) is indicated as black boxes. Numbers indicate positions of amino acids in the Epa protein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part on unexpected discoveries concerning a cell surface protein of the gram-positive bacterium *Erysipelothrix rhusiopathiae*. In particular, evidence suggested that, unexpectedly, the N-terminal portion of this protein was protective. Indeed, an N-terminal polypeptide was found to confer protection from *E. rhusiopathiae* infection when used to vaccinate mice and pigs.

The gene encoding a protective antigen of *E. rhusiopathiae,* the causative agent of erysipelas in animals and erysipeloid in humans, was cloned and sequenced. The gene encodes a mature polypeptide of 597 amino acids, plus a putative signal sequence of 29 amino acids, resulting in a mature protein with a deduced molecular weight of 69,017 Da. The antigen was designated Epa (Erysipelas Protective Antigen).

Nearly one third of the Epa protein, at the C-terminal region, consists of a series of highly conserved 20-amino-acid repeats, showing structural and sequence similarities to C-terminal region of pneumococcal surface protein A (PspA) and other choline-binding proteins from *Streptococcus pneumoniae*. In a specific embodiment, there are nine repeats.

Because of its similarity to SpaA (Makino *et al.*, Microb. Patholg. 25:101-109, 1998), the antigen can be referred to as SpaA.1. However, unlike SpaA, SpaA.1 has nine repeats of a 20-amino acid sequence in the C-terminus (SpaA has only eight). Moreover, the data reported here represent the first confirmation that purified SpaA/SpaA.1, and particularly the N-terminal portion of the protein, can be used in an effective vaccine against *E. rhusiopathiae*, *e.g.*, to protect animals from erysipelas and humans from erysipeloid. Thus, the term "protective antigen" is believed to reflect the significance of the present invention.

Both the mature Epa protein and the C-terminal repeat region, but not the N-terminal segment, were expressed in *Escherichia coli* at 37°C, and purified as a histidine-tagged fusion recombinant protein. The N-terminal segment was expressed at 28°C and purified with the His-tag. Rabbit antiserum raised against the mature Epa

protein passively protected mice from lethal challenge with a virulent homologous strain, Fujisawa-SmR, suggesting that protection is mediated by a humoral antibody.

To initially determine which domain of the Epa protein is responsible for the observed protection, mice were actively immunized with either the mature Epa protein or the C-terminal repeat region and then challenged with Fujisawa-SmR. The result showed that mice immunized with the mature Epa protein, but not the C-terminal repeat region, were protected, suggesting that the protection-eliciting epitope(s) are located within the N-terminal two thirds of the Epa molecule. This result was confirmed by passive immunization experiments in which the protective activity of rabbit antiserum raised against mature Epa protein was not abolished by absorption with the purified recombinant C-terminal repeat region. In addition, antibody specific for the C-terminal repeat region was unable to protect mice from lethal challenge.

In Western immunoblotting experiments, the anti-Epa rabbit serum detected a prominent 69-kDa band in detergent-solubilized cell surface antigen preparations from all *E. rhusiopathiae* strains tested.

Finally, as reported above, the N-terminal portion expressed from a plasmid encoding amino acid residues 12 to 195 (SEQ ID NO:12) was found to protect mice and pigs from a lethal bacterial challenge.

Based on these results, the N-terminal one-third portion of Epa has been identified as containing an immuno-protective epitope, which can be introduced as a polypeptide immunogen and used in vaccine regimens in animals, particularly pigs and domestic foul (turkeys and chickens), or in people exposed to *E. rhusiopathiae*. The availability of a subunit vaccine is highly advantageous for a number of reasons. First, the vaccine composition need not include potential toxins, inflammatory components, and cross-reactive antigens from attenuated or killed bacteria. This may be critical to avoid side effects, such as an increase in arthritis lesions, associated with currently available vaccines. Second, the vaccine is inexpensive to prepare. Third, the vaccine can be delivered in a vector, a highly efficient vaccination strategy, which is simply not possible with a whole bacteria vaccine. Fourth, quality control and the actual composition of the vaccine can be controlled, in contrast to a whole bacteria or even a bacterial extract vaccine. The

following sections of the application more completely describe the various aspects of the invention, including the immuno-protective epitope and constructs for expression of polypeptide immunogens comprising the immuno-protective epitope; expression systems for *in vitro* production of the polypeptide immunogen (*e.g.*, fermentation) or *in vivo* delivery of the polypeptide immunogen by gene therapy (*i.e.*, a DNA vaccine); and vaccine technology (including a subunit polypeptide vaccine with an adjuvant, DNA vaccination, targeted vaccine delivery for systemic and mucosal immunity, and passive vaccination). The various headings (bold, underlined text), subheadings (bold italic, underlined text), and sections are provided for convenience, and are not intended to limit the scope of the invention.

The Immuno-protective Epitope of *E. rhusiopathiae*

The present invention provides an immuno-protective epitope of the Epa protein of *E. rhusiopathiae*. This epitope is about a 25 amino acid segment located in the N-terminal portion of Epa, *i.e.*, within residues from about 30 to about 447 of SEQ ID NO:2, preferably within residues from about 30 to about 195 of SEQ ID NO:2, and more preferably within residues from about 30 to about 100 of SEQ ID NO:2. In specific embodiments, convenient constructs encoding the immuno-protective epitope of Epa can be prepared by selecting coding sequences for the N-terminal portion of Epa with various restriction endonucleases, *e.g.*, *Bam*HI, *Kpn*I, and *Sac*I, for expression of polypeptide or preparation of a DNA vaccine vector. In another embodiment, expressed Epa can be cleaved proteolytically, *e.g.*, with pepsin, clostripain, trypsin, and the like, and the N-terminal immuno-protective fragment isolated or purified for use in a polypeptide vaccine.

The term "immuno-protective epitope" refers to a sequence in the Epa protein that elicits a protective immune response when administered to an animal. An N-terminal portion, particularly N-terminal one-third portion, of Epa contains the immuno-protective epitope. Similarly, an N-terminal immuno-protective fragment contains the epitope. The protective immune response generally involves both cellular and humoral immunity. In the present case, a strong antibody response to the epitope appears to be necessary for protection, as demonstrated by passive immunization experiments. Thus, the epitope can be associated with another

polypeptide containing a strong T cell epitope to generate a high antibody titer to the immuno-protective epitope.

The immuno-protective epitope should be delivered to a subject as an immunogenic polypeptide. As used herein, the term "immunogenic" means that the polypeptide is capable of eliciting a humoral or cellular immune response, and preferably both. An immunogenic polypeptide is also antigenic. A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains an epitope of at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a polypeptide, also called herein the epitope, can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier polypeptide for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier. However, the present invention provides for joining the immuno-protective epitope or antigenic portion of Epa with a carrier to generate an immunogenic polypeptide.

The term "carrier polypeptide" as used herein refers to a protein or immunogenic fragment thereof that can be conjugated or joined with the immuno-protective epitope to produce an immunogenic polypeptide. Examples of carrier proteins include, but are by no means limited to, keyhole limpet hemocyanin (KLY), albumin, cholera toxin (discussed in greater detail below), heat labile enterotoxin (LT), and the like. While chemical cross-linking of a peptide comprising the immuno-protective epitope to the carrier polypeptide can be used to prepare an immunogenic polypeptide, preferably the two components are prepared as a chimeric construct for expression of a fusion polypeptide.

In addition, chimeric fusion polypeptides of the immunogenic polypeptide with an purification handle, such as FLAG or GST (for immunopurification), or a HIS-tag (for Ni-chelation purification), are contemplated.

Where the full length Epa is used as the immunogenic polypeptide, preferably it is free from bacterial components, *e.g.*, in distinction to vaccines comprising whole bacteria engineered to express the immunogen, such as *E. coli*.

For example, the immuno-protective polypeptide can be purified after recombinant expression, or it can be delivered by expression *in situ*, *i.e.*, by expression from a vector (a DNA vaccine).

In a specific embodiment, the immunogenic polypeptide is immunologically similar to an N-terminal region of Epa. The term "immunologically similar" means that the immunogenic polypeptide cross reacts with antibodies, and preferably with T cells, specific for the immuno-protective epitope of the N-terminal region of Epa as described above. Thus, the immunogenic polypeptide can comprise an immuno-protective epitope of Epa from any strain of *E. rhusiopathiae*, provided it is immunologically similar to the N-terminal region of Epa in SEQ ID NO:2. As shown in the Examples, a number of different species of *E. rhusiopathiae* have proteins that are immunologically similar to the N-terminal region of Epa.

It is possible, however, that some pathogenic strains of bacteria may not be inactivated by a vaccine comprising the immuno-protective epitope of Epa or an immunologically similar immunogenic polypeptide. Thus, in another embodiment, based on the identification of the immuno-protective epitope of Epa of SEQ ID NO:2, the present invention provides an immuno-protective epitope from an N-terminal portion of a different strain of pathogenic bacteria corresponding to the immuno-protective epitope of Epa of SEQ ID NO:2. As used herein, the term "corresponding to" refers to a homologous region of a different gene or protein. Because the location of the immuno-protective epitope has been identified, the corresponding location in a homologous protein from another bacterial strain can be readily identified on the basis of sequence similarity, and preferably on the basis of homology, even if there is no immunological similarity as defined above. Thus, the present invention provides a strategy for preparing a vaccine against any pathogenic strain of bacteria having a protein homologous to Epa.

In addition, the present invention permits use of various mutants, sequence conservative variants, and functional conservative variants of Epa, provided that the N-terminal portions of all such variants retain the required immuno-protective effect.

The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.* DNA, or any process, mechanism, or result of such a change.

This includes gene mutations, in which the structure (*e.g.* DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.* protein) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.,* any kind of mutant.

"Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Allelic variants can be sequence-conservative variants.

"Function-conservative variants" are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Some allelic variations result in functional-conservative variants, such that an amino acid substitution does not dramatically affect protein function. Similarly, homologous proteins can be function-conservative variants. Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, more preferably at least 85%, and even more preferably at least 95%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared.

As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck *et al.*, Cell 50:667, 1987). Such proteins (and their encoding genes) have sequence homology, as reflected by their sequence similarity, whether in terms of percent similarity or the presence of specific residues or motifs.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck *et al.*, *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when a sufficient number of the nucleotides match over the defined length of the DNA sequences to differentiate the sequences from other sequences, as determined by sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, etc. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when enough of the amino acids are identical or similar (functionally identical) over a defined length to differentiate the sequences from other sequences. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA, etc.).

Furthermore, it should be noted that depending on the expression system employed, the expressed protein can differ from the predicted amino acid sequence encoded by a coding sequence. For example, as discussed below, a

construct for expression of the immunogenic polypeptide can express a protein comprising a signal sequence, which may be cleaved or not during cellular processing. In addition, other proteolytic cleavages may occure during expression. If the polypeptide is expressed in eukaryotic cells, it may be glycosylated if it contains a glycosylation site. Other possible changes include N-methylation, and the like.

Recombinant Expression Systems

The present invention contemplates various cloning and expression vectors for expression of the immunogenic polypeptides described herein. Such expression vectors can be used to transform cells *in vitro* to produce immunogenic polypeptides for protein vaccines, or *in vivo* to express the immunogenic polypeptide for a DNA vaccine.

The coding sequence for an immunogenic polypeptide may, and preferably do, include a signal sequence, which can be the endogenous signal sequence or fragment thereof of Epa, or can be a heterologous signal sequence, *e.g.*, for optimized signal sequence processing in a yeast, insect, or mammalian cell. The term "signal sequence" is used herein to refer to the N-terminal, hydrophobic sequence found on most secreted proteins that identifies it for processing for secretion from the cell. Generally, the signal sequence is cleaved during processing. However, various constructs of the invention can include a partial signal sequence (for example, residues 12-29 rather than 1-29 as exemplified, *infra*). It is not necessarily the case that the partial signal sequence is processed normally, or that it even provides for translocation during expression, *e.g.*, to the bacterial periplasm.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell*

Culture [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "nucleic acid molecule" (or alternatively "nucleic acid") refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. This term includes double-stranded DNA found, *inter alia*, in linear (*e.g.*, restriction fragments) or circular DNA molecules, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

The introduced gene or coding sequence may also be called a "cloned", "foreign", or "heterologous" gene or sequence, and may include regulatory or control

sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function.

The coding sequences herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications.

The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or functional assays, as described *infra*. A host cell has been "tansfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exongenous or heterologous DNA when the transfected DNA is expressed and effects a function or phenotype on the cell in which it is expressed. The term "expression system" means a host cell transformed by a compatible expression vector and cultured under suitable conditions *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

Proteins and polypeptides can be made in the host cell by expression of recombinant DNA. As used herein, the term "polypeptide" refers to an amino acid-based polymer, which can be encoded by a nucleic acid or prepared synthetically. Polypeptides can be proteins, protein fragments, chimeric proteins, etc. Generally, the term "protein" refers to a polypeptide expressed endogenously in a cell, *e.g.*, the naturally occurring form (or forms) of the amino acid-based polymer. Generally, a DNA sequence having instructions for a particular protein or enzyme is "transcribed" into a corresponding sequence of RNA. The RNA sequence in turn is "translated"

into the sequence of amino acids which form the protein or enzyme. An "amino acid sequence" is any chain of two or more amino acids.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" or "operatively associated with" of transcriptional and translational (*i.e.*, expression) control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself , *e.g.* the resulting protein, may also be said to be "expressed" by the cell.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.*, a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.*, transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having

inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is a such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, an gene is heterologous to the recombinant vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in which it is expressed, *e.g.*, a CHO cell.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, *supra*). For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook *et al.*, *supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

Expression Vectors

A wide variety of host/expression vector combinations (*i.e.*, expression systems) may be employed in expressing the immunogenic polypeptides of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, Gene 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; gram positive vectors such as *Strep. gardonii*; phage DNAS, *e.*g., the numerous derivatives of phage l, *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2m plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Expression of the protein or polypeptide may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, Cell 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner *et al.*, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445, 1981), the regulatory sequences of the metallothionein gene (Brinster *et al.*, Nature 296:39-42, 1982); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Komaroff, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731, 1978), or the *tac* promoter (DeBoer, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 80:21-25, 1983); see also "Useful proteins from recombinant bacteria" in Scientific American, 242:74-94, 1980; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and control regions that exhibit hematopoietic tissue specificity, in particular: beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, Nature 315:338-340, 1985; Kollias *et al.*, Cell 46:89-94, 1986),

hematopoietic stem cell differentiation factor promoters, erythropoietin receptor promoter (Maouche *et al.*, Blood, 15:2557, 1991), etc; and control regions that exhibit mucosal epithelial cell specificity.

Preferred vectors, particularly for cellular assays *in vitro* and vaccination *in vivo* or *ex vivo*, are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, and other recombinant viruses with desirable cellular tropism. Thus, a vector encoding an immunogenic polypeptide can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and vaccination procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*see, e.g.*, Miller and Rosman, BioTechniques, 7:980-990, 1992). Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. Preferably, the replication defective virus is a minimal virus, *i.e.*, it retains only the sequences of its genome which are necessary for encapsidating the genome to produce viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), vaccinia virus, and the like. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt *et al.*, Molec. Cell. Neurosci. 2:320-330, 1991; International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* (J. Clin. Invest. 90:626-630, 1992; see also La Salle *et al.*, Science 259:988-990, 1993); and a defective adeno-associated virus vector (Samulski *et al.*, J. Virol. 61:3096-3101,

1987; Samulski *et al.*, J. Virol. 63:3822-3828, 1989; Lebkowski *et al.*, Mol. Cell. Biol. 8:3988-3996, 1988).

Various companies produce viral vectors commercially, including but by no means limited to Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City, CA; retroviral, adenoviral, AAV vectors, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA; adenoviral and AAV vectors), Genvec (adenoviral vectors), IntroGene (Leiden, Netherlands; adenoviral vectors), Molecular Medicine (retroviral, adenoviral, AAV, and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

Adenovirus vectors. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard *et al.*, Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC VR-800), for example). Various replication defective adenovirus and minimum adenovirus vectors have been described (WO94/26914, WO95/02697, WO94/28938, WO94/28152, WO94/12649, WO95/02697 WO96/22378). The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero *et al.*, Gene 101:195 1991; EP 185 573; Graham, EMBO J. 3:2917, 1984; Graham *et al.*, J. Gen. Virol. 36:59 1977). Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Adeno-associated viruses. The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide

spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques.

Retrovirus vectors. In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, 1983, Cell 33:153; Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, 1988, J. Virol. 62:1120; Temin *et al.*, U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein *et al.* Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Suitable packaging cell lines have been described in the prior art, in particular the cell line PA317 (US 4,861,719); the PsiCRIP cell line (WO 90/02806) and the GP+envAm-12 cell line (WO 89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences which may include a part of the

gag gene (Bender *et al.*, J. Virol. 61:1639, 1987). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retrovirus vectors can also be introduced by DNA viruses, which permits one cycle of retroviral replication and amplifies tranfection efficiency (*see* WO 95/22617, WO 95/26411, WO 96/39036, WO 97/19182).

Lentivirus vectors. In another embodiment, lentiviral vectors are can be used as agents for the direct delivery and sustained expression of a transgene in several tissue types, including brain, retina, muscle, liver and blood. The vectors can efficiently transduce dividing and nondividing cells in these tissues, and maintain long-term expression of the gene of interest. For a review, *see*, Naldini, Curr. Opin. Biotechnol., 9:457-63, 1998; *see also* Zufferey, *et al.*, J. Virol., 72:9873-80, 1998). Lentiviral packaging cell lines are available and known generally in the art. They facilitate the production of high-titer lentivirus vectors for gene therapy. An example is a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate virusparticles at titers greater than 106 IU/ml for at least 3 to 4 days (Kafri, *et al.*, J. Virol., 73: 576-584, 1999). The vector produced by the inducible cell line can be concentrated as needed for efficiently transducing nondividing cells *in vitro* and *in vivo*.

Non-viral vectors. In another embodiment, the vector can be introduced *in vivo* by lipofection, as naked DNA, or with other transfection facilitating agents (peptides, polymers, etc.). Synthetic cationic lipids can be used to prepare liposomes for in vivo transfection of a gene encoding a marker (Felgner, et. al., Proc. Natl. Acad. Sci. U.S.A. 84:7413-7417, 1987; Felgner and Ringold, Science 337:387-388, 1989; see Mackey, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85:8027-8031, 1988; Ulmer *et al.*, Science 259:1745-1748, 1993). Useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey, *et al.*, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931), peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931).

It is also possible to introduce the vector in vivo as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun (ballistic transfection), or use of a DNA vector transporter (see, e.g., Wu *et al.*, J. Biol. Chem. 267:963-967, 1992; Wu and Wu, J. Biol. Chem. 263:14621-14624, 1988; Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams *et al.*, Proc. Natl. Acad. Sci. USA 88:2726-2730, 1991). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al.*, Hum. Gene Ther. 3:147-154, 1992; Wu and Wu, J. Biol. Chem. 262:4429-4432, 1987). US Patent Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal. Recently, a relatively low voltage, high efficiency *in vivo* DNA transfer technique, termed electrotransfer, has been described (Mir *et al.*, C.P. Acad. Sci., 321:893, 1998; WO 99/01157; WO 99/01158; WO 99/01175).

Vaccine Technology

As noted above, the present invention contemplates polypeptide vaccines, DNA vaccines, and passive immunization to prevent or treat an *E. rhusiopathiae* infection, or an associated disease (erysipelas or erysipeloid).

The vaccines of the invention are broadly applicable to protect an animal from infection by *E. rhusiopathiae*. The term "protect" is used herein to mean for the treatment or prevention of *E. rhusiopathiae* infection, and the diseases erysipelas and erysipeloid. Thus, any animal susceptible to this type of infection, including mammals and birds, particularly fowl, can be vaccinated. Most importantly, swine and turkeys, can be treated with a vaccine of the invention to prevent or treat erysipelas. Both of these animals are highly susceptible to erysipelas, which has a significant adverse impact on farmers who raise these animals. In addition, subunit

vaccines of the invention can also be prepared for administration to humans, particularly individuals who have close contact with swine and fowl, and thus have greater susceptibility to develop erysipeloid. Indeed, a subunit vaccine of the present invention, particularly one shown to be effective in pigs, is particularly desirable, as the side effects of current vaccines make them undesirable for humans.

Polypeptide Vaccines

As used herein, the term "polypeptide vaccine" refers to a vaccine comprising an immunogenic polypeptide and, generally, an adjuvant. The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Alternatively, or in addition, immunostimulatory proteins, as described below, can be provided as an adjuvant or to increase the immune response to a vaccine. Preferably, the adjuvant is pharmaceutically acceptable.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Sterile water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable

solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

DNA Vaccines

The term "DNA vaccines" is an informal term of art, and is used herein to refer to vaccines delivered by means of a recombinant vector. An alternative, and more descriptive term used herein is "vector vaccine" (since some potential vectors, such as retroviruses and lentiviruses are RNA viruses, and since in some instances non-viral RNA instead of DNA can be delivered to cells). Generally, the vector is administered *in vivo*, but *ex vivo* transduction of appropriate antigen presenting cells, such as dendritic cells, with administration of the transduced cells *in vivo*, is also contemplated. The vector systems described above are ideal for delivery of a vector for expression of an immunogenic polypeptide of the invention.

Vaccination Strategies

Various strategies can be employed to vaccinate subjects against *E. rhusiopathiae* infection. The polypeptide vaccine formulations can be delivered by subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.), subdermal (s.d.), intradermal (i.d.), or by administration to antigen presenting cells *ex vivo* followed by administration of the cells to the subject.

Similarly, any of the gene delivery methods described above can be used to administer a vector vaccine to a subject, such as naked DNA and RNA delivery, *e.g.*, by gene gun or direct injection.

Vaccination effectiveness may be enhanced by co-administration of an immunostimulatory molecule, such as an immunostimulatory or immunopotentiating, cytokine, lymphokine, or chemokine with the vaccine, particularly with a vector vaccine. For example, cytokines or cytokine genes such as interleukin (IL)-1, IL-2, IL-3, IL-4, IL-12, IL-13, granulocyte-macrophage (GM)-colony stimulating factor (CSF), macrophage inflammatory factor, as well as some key costimulatory molecules or their genes (*e.g.*, B7.1, B7.2) can be used.

Mucosal Vaccination. Mucosal vaccine strategies are particularly effective for many pathogenic bacteria, since infection often occurs via the mucosa. Indeed, the oral mucosa is strongly implicated in swine erysipelas. Thus, mucosal vaccination strategies for both polypeptide and DNA vaccines are contemplated.

While the mucosa can be targeted by local delivery of a vaccine, various strategies have been employed to deliver immunogenic proteins to the mucosa (these strategies include delivery of DNA vaccines as well, *e.g.*, by using the specific mucosal targeting proteins as vector targeting proteins, or by delivering the vaccine vector in an admixture with the mucosal targeting protein).

For example, in a specific embodiment, the immunogenic polypeptide or vector vaccine can be administered in an admixture with, or as a conjugate or chimeric fustion protein with, cholera toxin, such as cholera toxin B or a cholera toxin A/B chimera (Hajishengallis *et al.*, J Immunol.,154:4322-32, 1995; Jobling and Holmes, Infect Immun., 60:4915-24 , 1992). Mucosal vaccines based on use of the cholera toxin B subunit have been described (Lebens and Holmgren, Dev Biol Stand 82:215-27, 1994). In another embodiment, an admixture with heat labile enterotoxin (LT) can be prepared for mucosal vaccination.

Other mucosal immunization strategies include encapsulating the immunogen in microcapsules (U.S. Patents No. 5,075,109, No. 5,820,883, and No. 5,853,763) and using an immunopotentiating membranous carrier (WO 98/0558). Immunogenicity of orally administered immunogens can be enhanced by using red blood cells (rbc) or rbc ghosts (U.S. Patent No. 5,643,577), or by using blue tongue antigen (U.S. Patent No. 5,690,938). Systemic administration of a targeted immunogen can also produce mucosal immunization (*see*, U.S. Patent No. 5,518,725).

Various strategies can be used to deliver genes for expression in mucosal tissues, such as using chimeric rhinoviruses (U.S. Patent No. 5,714,374), adenoviruses, or specific targeting of a nucleic acid (WO 97/05267).

Passive Immunization

In addition to the active immunization vaccination strategies described above, the present invention further contemplates passive immunization with antibody reactive with, and preferably generated against, the immuno-protective epitope. As shown in Example 1, such an antibody (but not an antibody reactive with the C-terminal portion of Epa) protects mice from a bacterial challenge. Passive immunization is particularly effective for an incipient or established infection, before the host's immune system can respond.

One source of antibodies for use in passive immunization is from convalescent serum of affected animals of the same species as the infected host. Thus, for example, antibodies from pig sera can be isolated, preferably by affinity purification against the immuno-protective epitope, and used to passively immunize newly infected pigs.

Alternatively, antibodies can be generated against the immunogenic polypeptide, *i.e.*, the vaccine strategy can also be used to generate antibodies for passive immunization.

Antibodies to the Immuno-protective Epitope. According to the invention, the immunogenic polypeptide may be used as an immunogen to generate antibodies that recognize the the immuno-protective epitope. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. The anti-the immuno-protective epitope antibodies of the invention may be cross reactive, *e.g.*, they may recognize the immuno-protective epitope from different species. Polyclonal antibodies have greater likelihood of cross reactivity.

Various procedures known in the art may be used for the production of polyclonal antibodies to the immuno-protective epitope polypeptide or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the immunogenic polypeptide, including but not limited to rabbits, mice, rats, sheep, goats, etc. Preferably, the immunized animal is of the same species as the animal who will receive the antibodies in passive immunization, to avoid allergic reactions to the antibodies.

For preparation of monoclonal antibodies directed toward the immuno-protective epitope, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (Nature 256:495-497, 1975), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today 4:72, 1983; Cote et al., Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). In an additional embodiment of

the invention, monoclonal antibodies can be produced in germ-free animals (International Patent Publication No. WO 89/12690, published 28 December 1989). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., J. Bacteriol. 159:870, 1984; Neuberger et al., Nature 312:604-608, 1984; Takeda et al., Nature 314:452-454, 1985) by splicing the genes from a mouse antibody molecule specific for an the immuno-protective epitope polypeptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves. The same principles apply, of course, to treatment of any subject.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778) can be adapted to produce the immuno-protective epitope polypeptide-specific single chain antibodies. Indeed, these genes can be delivered for expression *in vivo*. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., Science 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for an the immuno-protective epitope polypeptide, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab¢ fragments which can be generated by reducing the disulfide bridges of the $F(ab¢)_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion

assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of an the immuno-protective epitope polypeptide, one may assay generated hybridomas for a product which binds to an the immuno-protective epitope polypeptide fragment containing such epitope. For selection of an antibody specific to an the immuno-protective epitope polypeptide from a particular species of animal, one can select on the basis of positive binding with the immuno-protective epitope polypeptide expressed by or isolated from cells of that species of animal.

Immunoassay for Protective Immunity to *E. rhusiopathiae*

In another embodiment, an immunogenic polypeptide of the invention, such as an immuno-protective N-terminal fragment of Epa, or any peptide containing the immuno-protective epitope, can be used in an immunoassay to detect protective antibodies against *E. rhusiopathiae* in an animal (*i.e.*, a subject), particularly a mammal or bird (fowl), and preferably in a pig, turkey, or human. Based on the discoveries of the present invention, a high titer of antibody reactive with (specific for) the immuno-protective epitope indicates that the individual may be protected from *E. rhusiopathiae* infection. Low or no detectable antibodies reactive with the immuno-protective epitope indicates that the individual may not be protected from infection.

The immunoassay of the invention can be used to detect antibody levels in subjects who have been exposed to *E. rhusiopathiae*, *e.g.*, in convalescent serum. It can also be used to detect antibodies in subjects of unknown status. High level antibody titers in such subjects would indicate prior exposure, and possibly

protective immunity, to *E. rhusiopathiae*. Finally, the immunoassay can be used to evaluate the effectiveness of a vaccine of the invention.

Any of the immunoassay formats described above can be used in an immunoassay of the invention. Preferably, an ELISA assay is used in which a polypeptide comprising the immuno-protective is adsorbed to the solid phase, sera (preferably in serial dilution) is contacted with the solid phase, and antibody binding is detected, *e.g.*, with a labeled antibody specific for antibodies in the serum. Alternatively, a competitive ELISA format could be used, in which immuno-protective antibodies in the serum sample compete for binding to the solid phase polypeptide against labeled antibodies specific for the epitope, *e.g.*, prepared as described above. In another alternative, the polypeptide comprising the epitope is labeled, and antibody specific for the polypeptide adsorbed to the solid phase support. The presence of antibodies in the biological sample (*e.g.*, serum) will result in competition for the polypeptide, preventing binding of the label to the adsorbed antibodies. In addition, convenient chromatographic immunoassay formats, as desribed below, can be used.

Although the immunoassays described here refer to testing for the presence of anti-immuno-protective antibodies in serum, any biological sample that provides antibodies, can be tested, including without limitation, blood, serum, plasma, tissue samples, lymph, mucosal secretions, sputum, synovial fluid and other inflammatory fluids, and the like.

Kits

The components for practicing the immunoassays can be conveniently provided in a kit form. In its simplest embodiment, a kit of the invention provides a polypeptide comprising the immuno-protective epitope and an antibody detector, such as a labeled antibody specific for antibodies from the subject to be tested. The amounts of each can be pre-measured to provide a specified number of assays.

In a further embodiment, the kit will include an assay container, such as a plate, preferably of plastic or a material treated to avoid non-specific binding of protein. As used herein, the term container has its broadest meaning, *i.e.*, any receptacle for holding material or reagents. It can be fabricated from glass, plastic, ceramic, metal, etc.

In still a further embodiment, the kit includes an immunochromatographic membrane or support, to which one reagent, either a polypeptide comprising the immuno-protective epitope or an antibody specific for the immuno-protective epitope, has been irreversibly coupled. Numerous methods and devices known in the art for immunochromatographic assays can be employed in the invention. Immunochromatographic assays are particularly useful under field conditions, where laboratory equipment is not available. Examples of such assays are provided in U.S. Patents No. 5,248,619, No. 5,451,504, No. 5,500,375, No. 5,624,809, and No. 5,658,801.

A kit of the invention preferably includes packaging and instructions for its use, *e.g.*, on the packaging or package insert.

EXAMPLES

The present invention will be better understood by reference to the following examples, which are provided by way of exemplification and are not intended to limit the invention.

EXAMPLE: Identification of Immuno-protective Region of a Cell Surface Protein of *Erysiplothrix rhusiopathiae*

To address the question of the nature of a protective antigen for *E. Rhusiopathiae*, we have analyzed cell surface protein antigens of the bacterium. This example describes the cloning and characterization of a protective protein antigen, termed Epa (erysipelas protective antigen), of *E. rhusiopathiae*, that is able to induce antibodies that protect against lethal challenge. We further show that the region responsible for protection is located within the N-terminal portion of the protein.

Materials and Methods

Bacterial strains and growth conditions. *E. rhusiopathiae* strains used were Fujisawa (Serovar 1a), Fujisawa-SmR (Serovar 1a) (Shimoji *et al.*, Infect. Immun., 62:2806-2810, 1994), 422/1E1 (Serovar 1b), ATCC 19414^T (Serovar 2), SE-9 (Serovar 2). These strains were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) medium containing 0.1% Tween 80 (pH7.6) (BHI-T80).

Escherichia coli strains were grown in Luria-Bertani (LB) medium. When appropriate, medium was supplemented with tetracycline (25μg/ml), ampicillin (100μg/ml), kanamycin (50μg/ml) or isopropyl-β-D-thiogalactopyranoside (IPTG) (1mM).

***Construction of genomic libraries of* E. rhusiopathiae.** Genomic DNA from *E. rhusiopathiae* strain Fujisawa was prepared as previously described (Galan and Timoney, Infect. Immun. 58:3116-3121, 1990). The genomic DNA was partially digested with *Sau*3A I and fractionated on a sucrose gradient to yield 3- to 5-kilobase (kb) fragments. The DNA fragments were ligated to *Bam*HI-predigested ZAP Express vector (Stratagene, La Jolla, CA) and *in vitro* packaging was conducted using Gigapack III Gold packaging extract (Stratagene) according to the manufacture's instruction.

Cloning of Epa gene. The genomic library was plated to give approximately 200 to 400 plaques per plate. The plates were overlaid with nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) previously soaked in 10mM IPTG and incubated at 37°C overnight. The membranes were incubated for 2 hours at room temperature with convalescent serum from a pig experimentally inoculated with the wild-type *E. rhusiopathiae* strain Fujisawa. After washing, the membranes were incubated for 2 hours at room temperature with peroxidase-labeled goat anti-pig immunoglobulin (Ig) G (Rockland, Inc., Gilbertsville, PA). Detection of the peroxidase-labeled goat anti-pig IgG was performed with 0.03% 3,3'-diaminobenzidine tetrahydrochloride dihydrate and 0.003 % hydrogen peroxide in phosphate-buffered saline (PBS). Immunoreactive plaques were isolated and used for further experiments.

For protection experiments, phage lysates from the positive clones that reacted with the pig antiserum were prepared for immunization as described previously (Galan and Timoney, *supra*, 1990). Briefly, groups of 13 BALB/c mice were immunized subcutaneously (s.c.) with the phage lysates emulsified with complete Freund adjuvant and then boosted s.c. with the same antigen preparation 2 and 3 weeks after first immunization. The mice were challenged s.c. with about 10 LD50 cells of Fujisawa-SmR strain 7 days after final boosting and deaths were recorded for 14 days. Two recombinant phage clones, which showed protective

activities, were subjected to *in vivo* excision to form recombinant phagemid pBK-CMV by using ExAssist helper phage and *E. coli* XLOLR system (Stratagene).

DNA sequencing and data analysis. Nucleotide sequencing of both strands of the cloned DNA in pBK-CMV was performed using the dideoxy chain termination method (Sanger *et al.*, Proc. Natl. Acad. Sci. USA, 74:5463, 1977) using fluorescent dye terminators and cycle sequencing reactions. Samples were electrophoresed, detected, and analyzed on an Applied Biosystems DNA sequencer. Initial sequencing reactions were performed with pBK-CMV vector primers T3 (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO:5) and T7 (5'-GTAATACGACTCACTATAGGGC-3') (SEQ ID NO:6). Subsequent primers were synthesized on the basis of sequence data previously obtained with vector primers. The sequences were analyzed with the GENETYX-MAC program, version 7.3 (SDC, Tokyo, Japan). Sequence similarity searches were performed with GenBank sequences by using BLAST network service.

Construction and purification of recombinant fusion proteins. Construction and purification of histidine (His)-tagged fusion protein was performed using the QIAexpress Kit (Qiagen Inc., Santa Clarita, CA). Insert DNA was amplified by PCR as previously described (Shimoji *et al.*, J. Clin. Microbiol., 36:86-89, 1998) from subclone plasmids of pERc6 (Figure 1), using primers constituting an in-frame *Bam*HI restriction site at the 5' end and T7 (5'-GTAATACGACTCACTATAGGGC-3') (SEQ ID NO:6) primers. Primers ORF-1 (5'-CATTGGATCCAGTCTTATGTCGTGCTTACT-3') (SEQ ID NO:7) and T7 were used to amplify *Epa* gene regions ranging from bp 34 to 1878, from bp 34 to 1329 and bp 34 to 587 from subclones pERc6.1, pERc6.3 and pERc6.4, respectively, ORF-1.1 (5'-CTCAGGATCCTAGTTCCTCTAAGAGATAGA-3') (SEQ ID NO:8) and T7 for the region ranging from bp 569 to 1329 from subclone pERc6.3, ORF-1.2 (5'-CATTGGATCCTCAAAAGAAGGG TGGATTA-3') (SEQ ID NO:9) and T7 for the region ranging from bp 1333 to 1878 from subclone pERc6.1, WG1 (5'-CCCCGGATCCTACAACAAAATGACTGATGC-3') (SEQ ID NO:10) and T7 for the region ranging from bp 178 to 1329 from subclone pERc6.3. These amplified products were digested with *Bam*HI and *Kpn*I, and then cloned in-frame into one of the expression vector pQE series to generate plasmids pQEr1 to pQEr6 (Figure 4).

Rabbit immunization. Purified recombinant Epa protein was run on sodium dodecyl sulfate (SDS)-12% polyacrylamide gel by the method of Laemmli (Laemmli, Nature, 227:680-685, 1970). The gel regions where the recombinant Epa protein band migrated were excised from the gel, ground, mixed with distilled water for preparation of antigen. New Zealand white rabbits (Charles River) were immunized s.c. at multiple sites with the gel fragments containing approximately 450μg of total protein. The rabbits were boosted once with the same gel preparation 30 days later and then bled 15 days after the boosting. Sera were filter sterilized and stored at -70°C until use.

Absorption of anti-Epa rabbit serum with the C-terminal Epa truncate. Approximately 3.5 mg of purified recombinant C-terminal region of Epa protein was coupled with 100 mg of beads (3M Emphaze Biosupport Medium; Pierce, Rockford, Ill.) according to the manufacture's instruction. As a control, the beads coupled with 3.5 mg of bovine serum albumin (BSA) were used. The anti-Epa rabbit serum (1.0 ml) was incubated under rotation with the protein-coupled beads at 4°C overnight. After separating the beads from the antiserum by centrifugation, the beads were washed three times with 10 ml of PBS (pH7.6) and then treated with 2 ml of 100 mM glycine/HCl buffer (pH 2.2) for elution of bound antibody specific for the C-terminal repeat region of Epa protein. The antibody solution was neutralized immediately after elution with 1 M Trizma Base. The beads were then washed three times with PBS and used for further absorption of the antiserum. These absorption procedures were repeated 3 times with the same 1.0 ml antiserum sample and the absorbed antiserum was used for Western immunoblot analysis and passive immunization experiments. The eluted antibody solution was pooled, dialyzed against PBS (pH7.6), concentrated to 1.0 ml and BSA was added to a final concentration of 1%. This final preparation was used for Western immunoblot analysis and passive immunization experiments.

Immunization experiments. Nine- to twelve-week-old female BALB/c mice (Charles River) were used. For passive immunization experiments, mice were injected intraperitoneally (i.p.) with either 0.1 ml of rabbit antiserum or 0.1 ml of eluted C-terminal region-specific antibodies (approximately 60 μg of protein). Eight hours after injection of the antiserum or the antibody, mice were

challenged with Fujisawa-SmR and were observed for clinical symptoms and death for 14 days. For active immunization experiments, mice were immunized i.p. with approximately 50 μg of purified recombinant protein emulsified with complete Freund adjuvant and then boosted s.c. with approximately 37 μg of the same protein emulsified with incomplete Freund adjuvant 3 weeks later. Seven days after boosting, mice were challenged with Fujisawa-SmR and were observed for clinical symptoms and death for 14 days.

Challenge inoculation. The strain Fujisawa-SmR was grown in BHI-T80 at 37°C for 14 hours and diluted with BHI-T80. Mice were inoculated with 0.1 ml of appropriate dilutions of the bacterial suspension of the bacteria.

Cell surface extract and supernatant preparation. Bacterial strains were grown in 10 ml of BHI-T80 for 14 hours at 37°C. The cultures were centrifuged, and the supernatants were filtered (0.22m pore size; Millipore Corp., Bedford, Mass.) and used for Western immunoblot analysis. For cell surface antigen preparation, the pelleted cells were treated with Triton X-100 as previously described (Galan and Timoney, *supra*, 1990; Lachmann and Deicher, Infect. Immun., 1986; Shimoji *et al.*, Infect. Immun., 66:3250-3254, 1998). Briefly, the cells were washed once with 20 mM Tris-HCl (pH7.6) and suspended in 0.5 ml of 20 mM Tris (pH7.6) containing 0.5% Triton X-100. The cells were incubated at 37°C for 1 hour with rotation. Cells were removed by centrifugation, and the supernatants were used for Western immunoblot analysis.

Western immunoblotting. For localization of Epa, a 7.5-μl volume of the supernatant fluids or the Triton X-100-solubilized surface antigen extracts were electrophoresed in SDS-12% polyacrylamide gel by the method of Laemmli, *supra*, 1970 and transferred to nitrocellulose membranes (Schleicher & Schuell). The nitrocellulose membranes were incubated for 1 hour in blocking solution, which contained 5% skim milk in TTBS (20mM Tris, 500mM NaCl, 0.05% Tween20 [pH7.5]) and then incubated for 1 hour with the anti-Epa rabbit serum diluted 1:500 in TTBS containing 5% skim milk. The membranes were washed in TTBS and then incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories, CA) diluted 1:2,000 in TTBS containing 5% skim milk. Bound antibodies were detected using chemiluminescence (ECL, Amersham).

For confirmation of absorption of anti-Epa rabbit serum with the C-terminal repeat region of Epa protein, nitrocellulose strips containing the C-terminal region fragment were incubated with various dilutions of the absorbed antiserum or the eluted antibody for 1 hour at room temperature. The bound antibodies were detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.).

Southern hybridization. Genomic DNAs from *E. rhusiopathiae* strains were digested with the restriction enzyme *Eco*RV and transferred to nitrocellulose membrane (Schleicher & Schuell). The PCR product used for construction of recombinant mature Epa protein was used for labeling as a probe. Subsequent hybridization and detection of the probe was performed using ECL direct nucleic acid labeling and detection systems (Amersham) according to the manufacture's instruction.

Results

Cloning of* epa *gene. The genomic DNA library of *E. rhusiopathiae* strain Fujisawa was screened with *E. rhusiopathiae* convalescent pig serum, and 22 positive clones were detected from a total of 5000 plaques. Of 22 positive clones, 5 clones which showed a strong positive signal were chosen and further analyzed for their protective activities. Groups of 13 mice immunized s.c. with phage lysates of each clone were challenged s.c. with 10 LD_{50} of virulent Fujisawa-SmR strain. Two clones (Clone 6 and Clone 20), which showed protective activities (85% and 92% survival respectively), were subsequently subjected to *in vivo* excision to generate phagemids pERc6 and pERc20, respectively. Restriction analysis of the insert in these phagemids showed the same restriction pattern, suggesting that inserts of the two clones are identical. Therefore, one clone (pERc6) was subjected to sequence analysis.

The DNA sequence of the insert of pERc6 revealed that the insert contains one complete open reading frame (ORF) and one incomplete ORF (Figure 1). To determine which ORF product is responsible for the protection, deletion clones of pERC6 were constructed. The subclones pERc6.1 and pERc6.2, which contain each ORF respectively, were obtained by deletion of the *Eco*RV and *Sca*I, and *Hinc*II and *Eco*RV fragments respectively (Figure 1). Lysates of *E. coli* DH5α containing each

subclone were examined in immunoblotting with the convalescent pig serum, and a positive reaction was detected in subclone pERc6.1 (data not shown), indicating that pERc6.1 contains the structural gene involved in the protection. The gene was designated *epa* (erysipelas protective antigen).

Analysis of the* epa *gene sequence. The sequences of the *epa* and upstream region are shown in Figure 2. The *epa* ORF is preceded by a potential ribosomal binding site (GGGGGA). Putative -35 (TTTACA) and -10 (TATTCT) regions were identified upstream of the ribosomal binding site.

The deduced amino acid sequence of Epa reveals that it encodes a protein of 626 amino acids with a potential signal-peptidase cleaving site, predicted by the method of von Heijne (Eur. J. Biochem., 133:17-21, 1983), between amino acids 29 and 30, resulting in a mature protein of 597 amino acids with a deduced molecular weight of 69,017 Da. This protein contains three distinct domains: (i) a 29-amino acid putative signal sequence, (ii) an N-terminal region (residues 30 through 447), which is mainly hydrophobic and has no obvious homology with proteins in the GenBank database except for SpaA of *E. rhusiopathiae* that was recently described by Makino *et al.*, *supra*, 1998, and (iii) a C-terminal region containing nine tandem repeats of 20 amino acids.

The C-terminal region shows both structural and sequence similarity with C-terminal region of choline-binding proteins of *Streptococcus pneumoniae*, such as PspA (Yother and Briles, J. Bacterial,174: 601-608, 1992), PcpA (Sanchez-Beato, et al., FEMS Microbiol Lett. 164:207-214, 1998), and CbpA (Rosenow, et al., Mol. Microbiol 25:819-829, 1997). PspA has been shown to be a protective antigen and plays an important role in virulence (McDaniel *et al.*, J. Exp. Med., 165:381-394, 1987). Amino acid sequence comparison between the C-terminal region of Epa and that of PspA shows that they are 53.5% identical over 170 residues (Figure 3). These choline-binding proteins of *S. pneumoniae* are located on the cell surface and the C-terminal repeat domain of these proteins are known to be involved in anchoring to the cell surface (Rosenow *et al.*, *supra;* Sanchez-Beato *et al.*, *supra* Yother *et al.*, J. Bacteriol., 174:610-618, 1992; and, Yother and White, J. Bacteriol., 176:2976-2985, 1994). Like these cell surface proteins of *S. pneumoniae*, neither the hexamer LPXTGX (SEQ ID NO:3) nor the highly hydrophobic membrane-spanning region,

which are important for anchoring surface proteins in many gram-positive bacteria (Fischetti *et al.*, Mol. Microbiol., 4:1603-1605, 1990), were found in the C-terminal region of Epa. However, an LPXTGX (SEQ ID NO:3) motif, LPGTGV (SEQ ID NO:11), was found in the N-terminal region of the Epa protein (21-amino acid from the beginning of the mature protein). A similar exception is also found in *S. pneumoniae* IgA1 protease (Wani *et al.*, Infect. Immun., 64:3967-3974, 1996). A proline-rich region, which is followed by the 10 tandem repeats in the C-terminal end of PspA (Yother and Briles, *supra*), was not observed in the sequence of Epa protein.

The repeat domain at the C-terminal end of the Epa protein also shows significant homology with the C-terminal region of glucan-binding protein of *Streptococcus mutans* (Banas *et al.*, Infect. Immun., 58:667-673, 1990) (25.5% identity over 145 residues), glucosyltransferase of *Streptococcus salivarius* (Simpson *et al.*, Infect. Immun., 63:609-621, 1995) (30.7% identity over 179 residues), *Streptococcus sobrinus* (Ferretti *et al.*, J. Bacteriol., 169:4271-4278, 1987) (28.2% identity over 181 residues), *Streptococcus downei* (Gilmore *et al.*, Infect. Immun., 58:2452-2458, 1990) (31.3% identity over 134 residues), and *Clostridium difficile* toxin A (Dove *et al.*, Infect. Immun., 58:480-488, 1990) (28.7% identity over 188 residues) and toxin B (Barroso *et al.*, Nucleic Acids. Res., 18:4004, 1990) (25.5% identity over 188 residues). These proteins are known to be secreted from these bacteria and play an important role in pathogenesis by binding to carbohydrates in host tissues via the C-terminal repeat domain (Wren, Mol. Microbiol., 5:797-803, 1991).

Comparison of the deduced amino acid sequence of Epa with SpaA (Makino *et al.*, *supra*, 1998) of *E. rhusiopathiae* shows that these proteins are nearly identical; differences were observed at residues 426 and 435, and in the C-terminal region of the protein, in which SpaA lacks one of the 20 amino acid repeats, resulting in a protein of 606 amino acids with eight tandem repeats.

Construction of His-tagged fusion proteins. To produce the recombinant Epa protein, the DNA fragment encoding the mature Epa was amplified from pERc6.1 and cloned into pQE30 expression vector. To produce Epa truncates, the subclones pERc6.3 and pERc6.4 which were constructed from pERc6 by deleting *Eco*RI fragments (Figure 1) and the DNA fragments which encode different regions

were amplified from these subclones and cloned into pQE expression vectors. The recombinant pQE plasmids were transformed into *E. coli* M15 and transformants were checked for the expression of fusion proteins by purification with chromatography on Ni-NTA resins followed by SDS-PAGE and Western immunoblot analyses.

The mature Epa protein and the its C-terminal repeat region (Figure 4) were efficiently expressed as His-tagged fusion proteins, which were purified on Ni-NTA resin and could be separated by SDS-PAGE. However, regions other than the C-terminal repeats could not be expressed in several different experiments using various conditions. We found that lysates of *E. coli* DH5α containing either the plasmid pERc6.3 or pERc6.4 were not reactive with convalescent pig serum.

The purified mature Epa protein gave a prominent 69-kDa band with a larger band at approximately 140-kDa and lower minor bands on SDS-PAGE. The gel regions where the prominent 69-kDa protein migrated were excised and injected into rabbits for the preparation of anti-Epa serum. When this antiserum was used in Western immunoblots with recombinant Epa, it was reactive with not only the 69-kDa protein but the 140-kDa band and lower molecular weight bands similar to those observed with expression of mature Epa protein (data not shown). Thus, we conclude that the larger and lower bands are a dimer and degradation products or processed forms of Epa, respectively. The purified C-terminal region of Epa gave a prominent 21-kDa band.

Passive immunization experiments. In *E. rhusiopathiae* infection, humoral as well as cellular immunity are involved in protection (Shimoji *et al.*, *supra*, 1998). To determine which immunity is responsible for the protection induced by Epa, passive immunization experiments were performed using the anti-Epa rabbit serum. Since the LD_{50} of *E. rhusiopathiae* Fujisawa-SmR varies according to the route of the challenge inoculation (s.c. LD_{50}, 16 colony forming units; i.p. LD_{50}, 3200 colony forming units [Shimoj, *et al.* Infect. Immunol. 66:3250-3254, 1998; Shimoji, et al. Infect. Immunol. 62:2806-2810, 1994]), protection experiments were conducted by both s.c. and i.p. inoculations. Eight hours after injection of 0.1 ml of the antiserum, mice were challenged with Fujisawa-SmR strain and observed for clinical symptoms and death for 14 days. As shown in Table 1, when challenged with 5 LD_{50} cells, all the antiserum treated mice survived without any clinical symptoms. When challenged

with 100 LD_{50} cells, half of the antiserum treated mice showed clinical symptoms, such as depression; single mice challenged s.c. died on days 4, 8, and 11, and single mice challenged i.p. died on days 8 and 14. All control mice died within 4 days after challenge. Thus, the anti-Epa serum passively protected mice, and the route of challenge inoculation did not affect the protective activity of the antiserum.

TABLE 1

Passive immunization of mice against virulent *E. rhusiopathiae* strain by anti-Epa rabbit serum*

Challenge route	Challenge dose	No. of survivors/total	
		Control	Anti-Epa serum
s.c.	5 LD_{50}	0/5	5/5*
	100 LD_{50}		7/10**
i.p.	5 LD_{50}	0/5	5/5*
	100 LD_{50}		8/10*

*Eight hours after injection of 0.1 ml of the anti-Epa rabbit serum, mice were challenged with virulent *E. rhusiopathiae* Fujisawa-SmR strain. Control mice were injected with 0.1 ml of preimmune serum. Asterisks indicate differences (*; $P<0.01$, **; $P<0.05$) as compared to control by the Fisher exact test.

Determination of the protective domain of the Epa protein. To examine whether the C-terminal repeat region of Epa protein alone can induce complete protection, mice were actively immunized with either the purified mature protein or the C-terminal repeat region and then challenged s.c. with 10 LD_{50} cells of the Fujisawa-SmR strain (Table 2). All the mice immunized with the mature Epa protein survived without any clinical symptoms, whereas all the control mice and 4 of the 5 mice immunized the C-terminal repeat region protein died within 4 days after challenge. This result strongly suggests that the C-terminal repeat region of Epa does not play a major role in protection and that the protection-eliciting epitope(s) of Epa are located with the N-terminal two thirds of the protein.

TABLE 2

Active immunization of mice against virulent *E. rhusiopathiae* strain by recombinant proteins

Immunization[a]	No. of survivors/total[b]
Control	0/4
Mature Epa	4/4*
C-terminal region	1/5

[a]Mice were immunized with recombinant protein with adjuvant and boosted 3 weeks later. Control mice were treated with PBS with adjuvant.

[b]Mice were challenged s.c. with 10 LD_{50} of the virulent *E. rhusiopathiae* Fujisawa-SmR stain. Asterisks indicate differences (*; $P<0.05$) as compared to control by the Fisher exact test.

The result that the C-terminal repeat region of the Epa protein does not play a major role in protection was further confirmed by passive immunization experiments. The protective anti-Epa rabbit serum was absorbed with the purified recombinant C-terminal repeat region of Epa and C-terminal region-specific antibodies were obtained. A 10^{-4} dilution of the absorbed serum was very weakly reactive with the recombinant C-terminal region of the Epa protein, however, the eluted C-terminal region-specific antibody was strongly reactive at a 10^{-5} dilution (these data were obtained by Western blotting of nitrocellulose strips containing the C-terminal region fragment incubated with antibody preparations and detected with an alkaline-phosphate-conjugated goat anti-rabbit IgG). These results suggest that the adsorbed anti-Epa rabbit serum was significantly reduced in C-terminal region-specific antibodies and that a high titer of C-terminal region-specific antibodies was obtained by eluting the bound antibody from the antigen-coupled beads.

Mice were then treated with either the absorbed antiserum or the eluted antibody and then challenge s.c. with 10 LD_{50} cells of Fujisawa-SmR (Table 3).

TABLE 3

Passive immunization of mice against virulent *E. rhusiopahtiae* strain by absorbed serum or C-terminal region-specific antibody[a]

Treatment	No. of survivors/total
Control	0/4
Anti-Epa serum absorbed with BSA	3/5
Anti-Epa serum absorbed with C-terminal region	4/5
C-terminal region-specific antibody	0/5

[a]Eight hours after injection of 0.1 ml of the absorbed serum or antibody, mice were challenged s.c. with 10 LD_{50} of the virulent *E. Rhusiopathiae* Fujisawa-SmR strain. Control mice were injected with 0.1 ml of PBS containing 1% BSA. Asterisks indicate differences (*; $P<0.05$), as compared to control by the Fisher exact test.

In the control group, all the mice died within 4 days after challenge. In the antiserum-treated groups, only one mouse treated with C-terminal region-absorbed antiserum died on day 4, and 2 mice died (days 7 and 9) in the BSA-absorbed antiserum treated mice. All the survivors did not show any clinical symptoms. Thus, mice treated with C-terminal region-absorbed antiserum were protected from lethal challenge, showing that absorption of anti-Epa serum with the C-terminal repeat region had little to no effect on protection. Furthermore, all the mice treated with the C-terminal region specific-antibody were not protected; 4 mice died within 4 days after challenge and the last mouse died on day 6. These results, taken together with the active immunization experiments, strongly suggest that the C-terminal repeat region of Epa protein does not play a major role in protection and that antibodies directed against the N-terminal region are responsible for protection.

***Presence of Epa in different strains of* E. rhusiopathiae.** To determine the molecular weight and the localization of the native Epa protein in different strains of *E. rhusiopathiae*, Western immunoblotting was performed with the anti-Epa rabbit serum. Cell surface antigen extracts prepared by treatment with Triton X-100 and unconcentrated culture supernatants from different strains (SE-9, ATCC 19414^T, 422/1E1, and Fujisawa-SmR) were analyzed for the presence of the antigen.

The antiserum detected a prominent 69-kDa band and multiple minor bands ranging in size between 32- and 66-kDa in the cell surface extracts from all the strains, suggesting that Epa is located on the cell surface of these different strains of *E. rhusiopathiae*. The antiserum also detected multiple bands in the unconcentrated culture supernatants from the strains. We found that an increase of the lower bands in the cell surface extracts and the culture supernatant corresponded to a reduction of the density of the 69-kDa band, suggesting that these lower bands are degradation products or normally processed forms of the 69-kDa protein. Preimmune rabbit serum was not reactive with any of these bands.

The presence of an *epa* gene in all four of these strains were confirmed by Southern hybridization using *epa* as a probe of *Eco*RV digested genomic DNAs of *E. rhusiopathiae*. The results showed that the probe hybridized with approximately 3.2 kb *Eco*RV fragment in the genomic DNAs from all the strains examined (data not shown).

Discussion

This study describes the cloning and characterization of the *epa* gene, which encodes the 69-kDa protective antigen of *E. rhusiopathiae*. The Epa protein was found to be expressed in all *E. rhusiopathiae* strains tested, representing three different serovars, and elicits protective antibodies in animals.

The antigen(s) responsible for protection against *E. rhusiopathiae* infection have not been well characterized. Recent studies on the protective antigens of the organism have drawn attention to the 66- to 64-kDa cell surface protein (Galan and Timoney, *supra*, 1990; Groschup *et al.*, Epidemiol. Infect., 107:637-649, 1991; Kitajima *et al.*, J. Vet. Med. Sci., 60:9-14, 1998; and, Sato and Saito, Vet. Microbiol., 43:173-182, 1995). Gálan and Timoney (*supra*, 1990) reported that the 66- to 64-kDa cell surface protein antigen in Triton X-100 extracts is a protective antigen of *E. rhusiopathiae*. In that study, mice immunized with a recombinant 66- to 64-kDa protein, which was fused with β-galactosidase, were poorly protected. They assumed that the incomplete protection may be derived from a less efficient protective immune response by the fused form of the protein with β-galactosidase. From this result, we hypothesized that in addition to the 66- to 64-kDa protein, other molecules on the cell surface may also be important for protective immunity. To test this hypothesis, we

isolated the clones expressing immunoreactive proteins by screening a ZAP Express genomic library of *E. rhusiopathiae* with a convalescent pig serum and further analyzed their protective activities in mice. Two protective clones were isolated and the inserts were found to be identical by restriction analysis. Although the sequence was not reported (Galan and Timoney, *supra*, 1990), the restriction enzyme map of the cloned gene was found to be different from the 66- to 64-kDa protein gene, indicating that these genes are different from each other. This result supports our hypothesis that other protein antigens are involved in protection against *E. rhusiopathiae*.

Recently, a cell surface protein gene (*spaA*) of *E. rhusiopathiae* was cloned by Makino *et al.* (*supra*, 1998). Comparison of the deduced amino acid sequence of *spaA* and Epa shows near identity. The differences are restricted to two amino acid changes in the region prior to the C-terminal repeat domain and the presence of an additional repeat domain in Epa. Because Epa is so similar to *spaA*, we have designated the Epa protective antigen as SpaA.1.

In that study (Makino *et al.*, *supra*, 1998), mice were inoculated with various recombinant *E. coli* carrying the *spaA* gene or its deletion derivatives and challenged with a virulent *E. rhusiopathiae* strain only 7 days after inoculation of the *E. coli*. They reported that only mice inoculated with the recombinant *E. coli* carrying the intact *spaA* gene were protected from lethal challenge. Although they concluded that the whole molecule, particularly the C-terminal repeat region, is essential for the observed protection, results with purified protein were not reported in their publication.

In the experiment disclosed here, the protective activity of Epa was examined by passive immunization with an anti-Epa rabbit serum prepared against purified Epa. Mice treated with this antiserum were protected from lethal challenge with 5 LD_{50} cells of the virulent strain, suggesting that the Epa alone was sufficient for protection against infection with low doses of challenge organisms. However, when the mice were challenged with a high dose (100 LD_{50}), half of the mice which were treated with anti-Epa rabbit serum showed clinical symptoms and 20 to 30% died. These results suggest that protective antibodies induced by immunization of a single antigen may not be sufficient for protection against infection with a high dose

of organisms. Either several different protective antigens or higher antibody titers, or both, may be required to induce complete protection with this dose of bacteria.

The protection-eliciting domain of the Epa protein was determined by active and passive immunization. In active immunization experiments, mice immunized with recombinant mature Epa protein, but not the C-terminal repeat region, were protected from lethal challenge with the virulent strain Fujisawa-SmR, showing that the C-terminal repeat region does not play a major role in protection. This was confirmed by passive immunization experiments in which the protective activity of the rabbit antiserum against the mature Epa protein could not be abolished by absorption with the recombinant C-terminal region and the specific antibody against this region was not protective. Taken together, these results strongly indicate that antibodies to the N-terminal two thirds of the Epa molecule are responsible for protection.

Sequence analysis of Epa revealed a repeat domain in the C-terminal region. Similar repeat domains have been observed in choline-binding proteins of *S. pneumoniae* (Rosenow *et al.*, *supra*, 1997; Sanchez-Beato *et al.*, *supra*, 1998; and Yother and Briles, *supra*, 1992). In these proteins, the repeat domain has been shown to be responsible for binding the choline moiety of teichoic acids found in the cell wall (Rosenow *et al.*, *supra*, 1997; Sanchez-Beato *et al.*, *supra*, 1998; Yother and Briles, *supra*, 1992; and, Yother and White, *supra*, 1994). Although the Epa protein was shown to be present in cell surface antigen preparations, its mechanism of attachment to the bacterial surface is currently unknown. However, since no teichoic acids have been described in the cell wall of *E. rhusiopathiae* (Mann, Zentralbl. Bakteriol. Orig., 290(0):510-522, 1969), it suggests the mechanism of attachment may be different from those of *S. pnuemoniae* surface proteins. Because Epa was found to lack the C-terminal anchor domain found in many surface proteins in gram-positive bacteria, its attachment to the cell surface may be by a novel mechanism for gram-positive bacteria.

It has been reported that the protective antigen of *E. rhusiopathiae* is also present in the culture supernatant of the organism and antiserum against the culture supernatant fluids is protective (Sato and Saito, *supra*, 1995; Sawada and Takahashi, Am. J. Vet. Res., 48:239-242, 1987; White and Verwey, Infect. Immunol.

1:380-386, 387-393, 1970). Although we found that Epa is also present in the culture supernatant, we do not yet know the relationship between the bound Epa protein and the protective antigens found in the culture supernatant reported so far (Sato and Saito, *supra*, 1995; Sawada and Takahashi, *supra*, 1987; White and Verwey, *supra*, 1970).

In Western and Southern blotting analysis, the *Epa* gene and its product are found to be present in all strains tested, suggesting that the protein is well conserved among *E. rhusiopathiae* bacteria. In erysipelas, the protective antigen has been known to be species-specific (Sawada and Takahashi, *supra*, 1987). The observation made here, that Epa is responsible for the species-specific protection, indicates that this protective antigen, particularly and surprisingly the N-terminal segment, is a good candidate for a new subunit vaccine against erysipelas.

EXAMPLE 2: Protective Effect with an N-Terminal Subunit Vaccine

An N-terminal segment (12-195) of Epa was expressed in *E. coli* and purified. The purified peptide was used to immunize mice, and the mice were subsequently challenged with *E. rhusiopathiae*. Mice immunized with full length Epa and the 12-195 N-terminal fragment were protected from the challenge dose of bacterial, but control animals were not. The sequence of the N-terminal portion of Epa that shows protection by active immunization is as follows:

Amino acid residues (12-195) of the Epa protein (SEQ ID NO:12) are:

SLMSCLLLTAMPLQTAFADSTDISVIPLIGEQVGLLPVLPGTGVHA
QEYNKMTDAYIEKLVSLINQKVKPFLINEPKGYQSFEAVNEEINSI
VSELKNEGMSLQNIHHMFKQSIQNLATRIGYRSFMQDAMYLENFE
RLTIPELDEAYVDLLVNYEVKHRILVKYEGKVKGRAPLEAFIVPLRD

Methods and Results

N-terminal region coding sequences were cloned into plasmid expression vectors for expression in *E. coli*. The *E. coli* clones were grown at 28°C, and a clone containing plasmid pQEr3 (containing a coding sequence for amino acid residues 12-195 of Epa) produced a small amount of recombinant protein. This

protein was purified and mice were immunized with the same quantity and immunization schedule as described for Epa (Example 1, *supra*).

Mice were challenged sub-cutaneously (s.c.) with approximately 70 LD50 cells of Fujisawa-SmR and observed for clinical symptoms and death for 14 days. As shown in Table 3, all 5 mice immunized with the 12-195 fragment of Epa protein survived without any clinical symptoms, whereas all 5 control mice died within 5 days after challenge. The arithmetic mean of ELISA IgG titers taken before bacterial challenge from individual sera of vaccinated mice was 76,800 against peptide 12-195 (using an optical density endpoint of 1.0).

TABLE 4

Protection of mice against challenge with a virulent *E. rhusiopathiae* strain after immunization with peptide 12-195 of the Epa (SpaA.1) protein

Immunization (a)	No. of survivors/total (b)	Anti-SpaA.1 IgG titer (c)
Control	0/5	< 100
Mature Epa	5/5*	66,560
Peptide 12-195	5/5*	76,800

Notes to Table 4:

(a) Groups of five BALB/c mice were immunized intraperitonealy (i.p.) with recombinant protein (50 μg) with complete Freund adjuvant and then boosted s.c. with the protein (40 μg) with incomplete Freund adjuvant 3 weeks later. Control mice were treated with PBS with adjuvant.

(b) Seven days after boosting, mice were challenged s.c. with approximately 70 LD_{50} cells of the virulent Fujisawa-SmR strain and were observed for clinical symptoms and death for 14 days.

(c) Six days after boosting, mice were bled. Anti-Epa titers represent the arithmetic mean of ELISA titers against the 12-195 peptide (using an optical density endpoint of 1.0) determined for individual sera.

* Asterisks indicate difference (*; $p<0.01$) as compared to control by the Fisher exact test.

Discussion

These results directly confirm the results shown in Example 1, that the N-terminal portion of Epa confers protection from bacterial challenge. Furthermore, the data demonstrate that purified protein can be used to effectively vaccinate animals against a lethal challenge dose of bacteria.

These data are in contrast to the data reported by Makino *et al.* (Microbial. Pathogenesis 25:101-109, 1998). The most important difference is the identification of the N-terminal portion, and not the C-terminal portion as reported in the publication, as the protective portion of Epa (termed Spa in Makino *et al.*). In addition, the protective effect reported by Makino *et al.* was achieved using a recombinant *E. coli* to immunize the animals, which left unresolved whether the purified protein would be effective.

Based on the data provided here, effective subunit vaccines, using recombinant protein produced in bacterial, yeast, or other high efficiency fermentation systems, can be prepared. Alternatively, DNA vaccines with expression vectors encoding the N-terminal or full length Epa protein can be prepared and delivered to animals. There is a significant need for this vaccine in the pig industry at the present time.

EXAMPLE 3: Immunogenicitiy of *Erysipelothrix rhusiopathiae* SpaA.1 Recombinant Protein in Pigs

The purpose of this example is to determine if *Erysipelothrix rhusiopathiae* pQER3 recombinant protein (SpaA.1 fragment) provides a protective effect in pigs challenged with virulent *E. rhusiopathiae.*

Materials and Methods

Immunization Experiments. Thirty-one pigs not previously exposed to *E. rhusiopathiae* were used in immunization experiments.

Ten animals per group were immunized intramuscularly (i.m.) with *E. rhusiopathiae* pQER3 purified recombinant protein emulsified in Fortasol®. For the primary dose, 2ml (200 μg) of the *E. rhusiopathiae* recombinant protein was administered and the animals were boosted three weeks later with 1 ml (100 μg) of the

recombinant protein in Fortasol®. In the second group, ten animals were immunized i.m. with 1 ml of monovalent *E. rhusiopathiae* bacteria, serotype 2 in an aluminum hydroxide gel using the same immunization schedule as the SpaA.1 animals. Also included was a control group of eleven animals, which were not immunized.

Serology. For serological analysis, blood was collected at day 0 (pre-vaccination), day 21 (2^{nd} vaccination) and 35 (day of challenge). The pig serum was separated, and serological responsiveness was determined by the presence of IgG antibodies using an *E. rhusiopathiae* antigen (whole cell lysate).

Challenge Inoculation. Fourteen days after the second vaccination, all pigs were challenged with 2 ml of a live, log-phase culture of *E. rhusiopathiae* strain E-1-6 (serotype 1A). *E. rhusiopathiae* was diluted 10^{-4} on sheep blood agar plates immediately following administration to the animals. Each pig received 4.9×10^6 cfu ml^{-1} of *E. rhusiopathiae* in the 2 ml challenge dose (2.45×10^6 cfu ml^{-1}). For three days pre-challenge and seven days post-challenge, the animals were monitored daily using rectal temperature. Animals were also monitored seven days post-challenge for clinical signs of infection. Any animals that died were necropsied and the spleen taken for reisolation of *E. rhusiopathiae*. Seven days post-challenge surviving pigs were sacrificed by stunning and exsanguination. A spleen sample was taken from each pig for reisolation of *E. rhusiopathiae*.

TABLE 5. Vaccination procedure.

Group	# Pigs	Vaccine	Dose	Route	Time
1	10	pQER3 + Fortasol	2 ml (200 μg) and 1 ml (100 μg)	1M 1M	1X (3-4 weeks of age) and 1X (6-7 weeks of age)
2	10	monovalent *E. rhusiopathiae* + aluminum hydroxide gel	1 ml and 1 ml	1M 1M	1X (3-4 weeks of age) and 1X (6-7 weeks of age)
3	11	None	n/a	n/a	n/a

Results

Thirty-one pigs were divided into groups and treated with the recombinant *E. rhusiopathiae* SpaA.1 subunit immunogen, monovalent whole organisms, or nothing (controls). Table 5 summarizes the vaccine procedure.

At day 35, the serological response in group 1 animal immunized with the *E. rhusiopathiae* pQER3 recombinant protein was greater than animals immunized with the monovalent *E. rhusiopathiae,* serotype 3 (see Table 6).

TABLE 6. Serology (reciprocal dilution antibody titre)

Group	Pig #1	1st Vaccination (Day 0)	2nd Vaccination (Day 21)	Challenge Day (Day 35)
1 (n = 10) pQER3 protein (Spa.A.1)	3838	640	>10240	>10240
	3841	5120	5120	>10240
	3842	>10240	640	>10240
	3843	160	160	>10240
	3844	640	1280	>10240
	3846	80	1280	>10240
	3848	640	2560	>10240
	3850	1280	320	1280
	3849	160	640	>10240
	3845	640	2560	>10240
2 (n=10) Monovalent Erysipelothrix	3847	5120	1280	5120
	3853	640	640	>10240
	3852	5120	2560	2560
	3851	2560	1280	5120
	3856	5120	5120	5120
	3855	5120	n/a[1]	n/a
	3854	10240	5120	5120
	3858	640	1280	5120
	3859	1280	320	5120
	3857	5120	1280	5120
3 (n = 11) Controls	3830	>10240	nd[2]	>10240
	3829	5120	nd	640
	3831	5120	nd	640
	3832	80	nd	1280
	3834	5120	nd	1280
	3833	5120	nd	5120
	3835	2560	nd	640
	3836	1280	nd	2560
	3837	5120	nd	5120
	3839	640	nd	n/a[3]
	3840	640	nd	1280

[1] Pig #3855 died prior to second vaccination
[2] Not determined.
[3] Pig # 3839 died prior to challenge.
n/a=non- applicable

The protective effect of *E. rhusiopathiae* pQER3 recombinant protein and monovalent *E. rhusiopathiae* serotype 3 vaccine was compared and evaluated according to a number of criteria such as fever or death, skin lesions, and reisolation

of *E. rhusiopathiae* from the spleen of the animal. 100% of the animals immunized with *E. rhusiopathiae* pQER3 recombinant protein and the monovalent *E. rhusiopathiae* serotype 3 vaccine, survived and were negative for febrile responses and skin lesions for two consecutive days (see Table 7).

TABLE 7. Protective effect of *E. rhusiopathiae* pQER3 recombinant protein, *E. rhusiopathiae,* monovalent serotype 3 and the control group on pigs challenged with virulent *E. rhusiopathiae.*

Group	Pig #	Fever[1] or Death	Skin Lesions	Re-isolation[2]	Combined Data
1 (n=10) pQER3 protein (Spa.A.1)	3838	-	-	-	-
	3841	-	-	-	-
	3842	-	-	-	-
	3843	-	-	+	+
	3844	-	-	-	-
	3846	-	-	-	-
	3848	-	-	-	-
	3850	-	-	+	+
	3849	-	-	-	-
	3845	-	-	-	-
2 (n=10) Monovalent Erysipelothrix	3847	-	-	+	
	3853	-	-	-	
	3852	-	-	-	
	3851	-	-	-	
	3856	-	-	-	
	3855	n/a	n/a	n/a	
	3854	-	-	-	
	3858	-	-	+	
	3859	-	-	-	
	3857	-	-	-	
				-	
3 (n=11) Controls	3830	-	-	+	+
	3829	-	-	-	-
	3831	-	-	nd[3]	nd
	3832	+ DIED[4]	+	+	+
	3834	-	-	-	-
	3833	-	-	-	-
	3835	-	-	-	-
	3836	+ DIED[4]	+	+	+
	3837	-	-	-	-
	3839	n/a[5]	n/a	n/a	n/a
	3840	+ DIED[4]	+	+	+

[1] Febrile response = temperature spikes (105.6°F or higher) for two consecutive days or more.

[2] Re-isolation of *Erysipelothrix rhusiopathiae* on Sheep Blood Agar plates.

[3] Pig #3831's spleen misplaced following necropsy.

[4] Pigs #3836 and #3832 died on Day 5 post-challenge. Pig #3840 died on Day 4 post-challenge.

[5] Pig #3839 died prior to challenge.

n/a=non- applicable

Conclusion

Animals in groups 1 and 2 were fully protected against heterologous challenge with virulent *E. rhusiopathiae*. However the serological response was higher in animals immunized with *E. rhusiopathiae* pQER3 recombinant protein. The higher serological response could be attributed to a more potent adjuvant, a highly immunogenic recombinant protein, or a combination of both. In contrast to the vaccinated animals, 40% of the control animals exhibited characteristic signs of *E. rhusiopathiae* infection. Thus, the subunit vaccine surprisingly demonstrates protective properties equal to the whole cell vaccine, with a much lower potential for adverse effects.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

All patents, patent applications, publications, and other materials cited herein are hereby incorporated herein reference in their entireties.

WHAT IS CLAIMED IS:

1. A vaccine comprising an immunogenic polypeptide of *Erysipelothrix rhusiopathiae*, wherein the immunogenic polypeptide comprises an immuno-protective epitope which is from an N-terminal region of an erysipelas protective antigen (Epa), and an adjuvant.

2. The vaccine of claim 1, wherein the immuno-protective epitope is about a 25 amino acid segment located in the N-terminal of Epa as depicted in SEQ ID NO:2 from about residue 30 to about residue 447.

3. The vaccine of claim 1, wherein the immuno-protective epitope is about a 25 amino acid segment located in the N-terminal of Epa as depicted in SEQ ID NO:2 from about residue 30 to about residue 195.

4. The vaccine of claim 1, wherein the immuno-protective epitope is about a 25 amino acid segment located in the N-terminal of Epa as depicted in SEQ ID NO:2 from about residue 30 to about residue 100.

5. The vaccine of claim 1, wherein the immunogenic polypeptide comprises a polypeptide having the sequence of SEQ ID NO:2 from about residue 30 to about residue 195.

6. The vaccine of claim 5, wherein the immunogenic polypeptide comprises a polypeptide having the sequence of SEQ ID NO:12.

7. The vaccine of claim 1, wherein the immunogenic polypeptide is a fusion polypeptide comprising the immuno-protective epitope and a purification handle or a carrier polypeptide.

8. A polypeptide comprising an amino acid sequence corresponding to about 25 contiguous residues of an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 447.

9. The polypeptide of claim 8 comprising an amino acid sequence corresponding to about 25 contiguous residues of an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 195.

10. The polypeptide of claim 8 comprising an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 195.

11. The polypeptide of claim 8 comprising an amino acid sequence as depicted in SEQ ID NO:12.

12. The polypeptide of claim 8 which is a fusion polypeptide comprising the immuno-protective epitope and a purification handle or a carrier polypeptide.

13. A nucleic acid encoding a polypeptide comprising an amino acid sequence corresponding to about 25 contiguous residues of, or about 85% identical to, an amino acid sequence of SEQ ID NO:2 from about amino acid residue 30 to about amino acid residue 447.

14. The nucleic acid of claim 13, wherein the polypeptide is a fusion polypeptide comprising the immuno-protective epitope and a carrier polypeptide.

15. The nucleic acid of claim 13, wherein the polypeptide is a fusion polypeptide comprising the immuno-protective epitope and a purification handle.

16. An expression vector comprising the nucleic acid of claim 13, wherein the nucleic acid is a DNA.

17. The expression vector of claim 16 which is a non-viral vector.

18. The expression vector of claim 16 which is a viral vector.

19. A method for protecting an animal from infection by *E. rhusiopathiae*, which method comprises administering an immunologically effective amount of the vaccine of claim 1 to the animal.

20. The method according to claim 19, wherein the animal is a turkey.

21. The method according to claim 19, wherein the animal is a pig.

22. A method for protecting an animal from infection by *E. rhusiopathiae*, which method comprises administering an immuno-protective amount of the expression vector of claim 16 to the animal.

23. The method according to claim 22, wherein the animal is a turkey.

24. The method according to claim 22, wherein the animal is a pig.

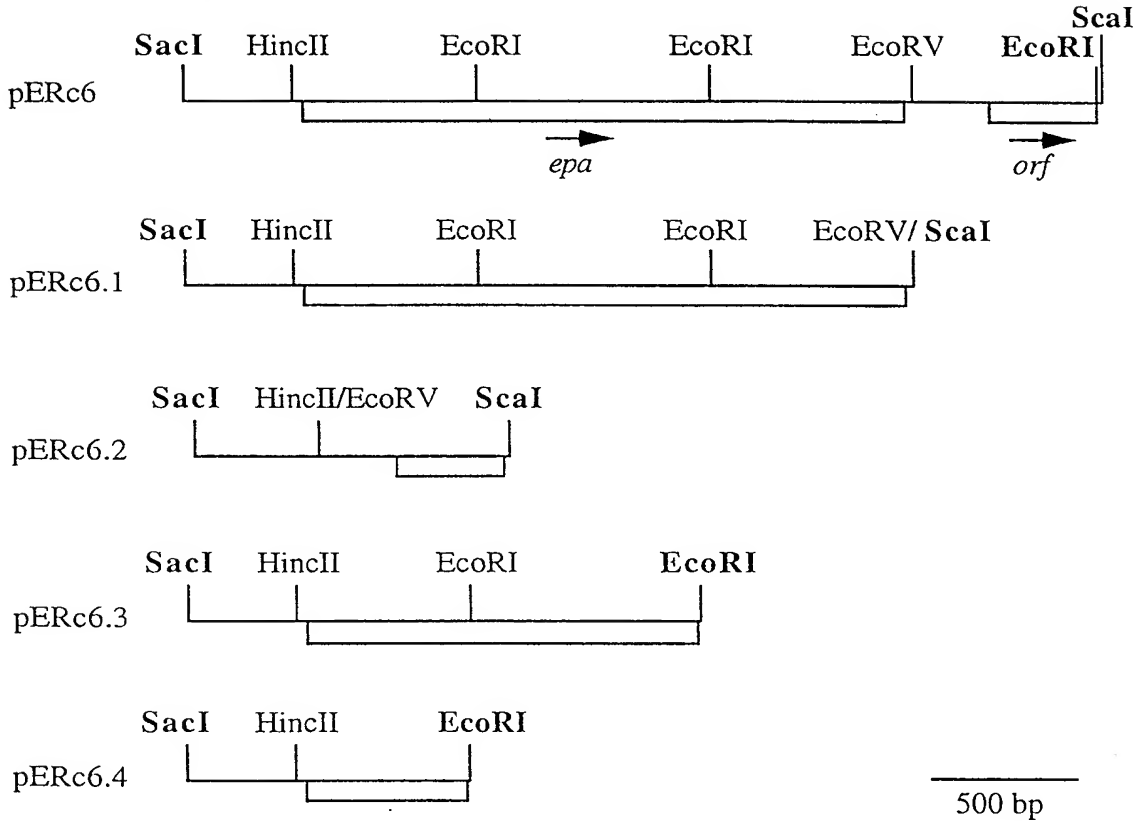
25. A method for protecting an animal from infection by *E. rhusiopathiae*, which method comprises administering an immuno-protective amount of an antibody that generated against or purified with an immuno-protective epitope which is from an N-terminal region of an erysipelas protective antigen (Epa) to the animal.

26. The method according to claim 25, wherein the immuno-protective epitope is about a 25 amino acid segment located in the N-terminal of Epa as depicted in SEQ ID NO:2 from about residue 30 to about residue 447.

27. The method according to claim 25, wherein the immuno-protective epitope is about a 25 amino acid segment located in the N-terminal of Epa as depicted in SEQ ID NO:2 from about residue 30 to about residue 195.

28. A method for detecting the presence of protective antibodies to *E. rhusiopathiae*, which method comprises detecting binding of antibodies from a biological sample with a polypeptide comprising an immuno-protective epitope of Epa protein of *E. rhusiopathiae* when the biological sample is contacted with the polypeptide under conditions that permit binding of antibodies in the biological sample with the polypeptide.

29. A kit for detecting the presence of protective antibodies to *E. rhusiopathiae*, which kit comprises a polypeptide comprising the immuno-protective epitope of EPA protein and an antibody detector.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EcoRI
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HincII/EcoRV
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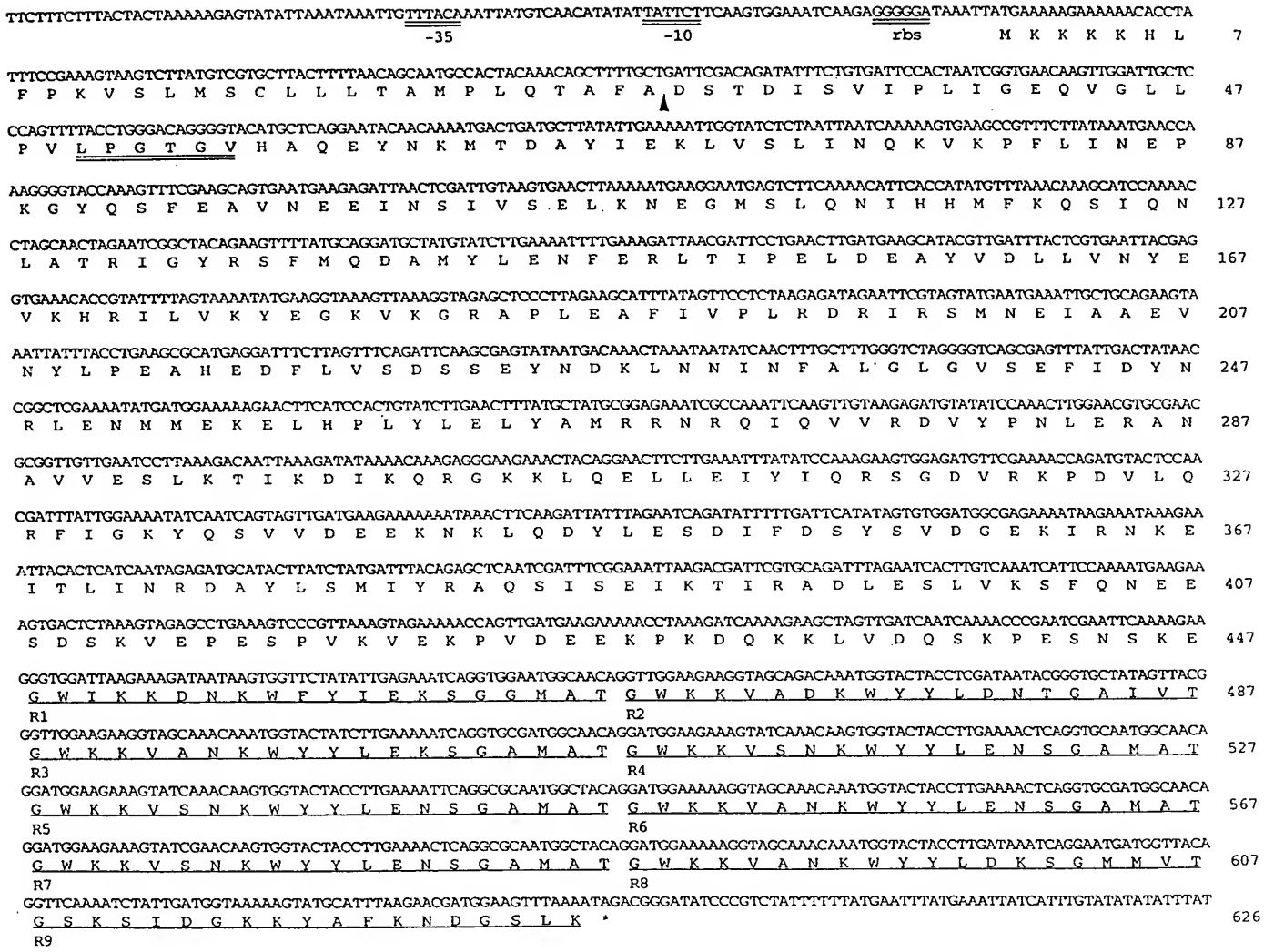
FIGURE 1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N Y L P E A H E D F L V S D S S E Y N D K L N N I N F A L G L G V S E F I D Y N 247
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R L E N M M E K E L H P L Y L E L Y A M R R N R Q I Q V V R D V Y P N L E R A N 287
GCGGTTGTTGAATCCTTAAAGACAATTAAAGATATAAAACAAAGAGGGAAGAAACTACAGGAACTTCTTGAAATTTATATCCAAAGAAGTGGAGATGTTCGAAAACCAGATGTACTCCAA
A V V E S L K T I K D I K Q R G K K L Q E L L E I Y I Q R S G D V R K P D V L Q 327
CGATTTATTGGAAAATATCAATCAGTAGTTGATGAAGAAAAAAATAAACTTCAAGATTATTTAGAATCAGATATTTTTGATTCATATAGTGTGGATGGCGAGAAAATAAGAAATAAAGAA
R F I G K Y Q S V V D E E K N K L Q D Y L E S D I F D S Y S V D G E K I R N K E 367
ATTACACTCATCAATAGAGATGCATACTTATCTATGATTTACAGAGCTCAATCGATTTCGGAAATTAAGACGATTCGTGCAGATTTAGAATCACTTGTCAAATCATTCCAAAATGAAGAA
I T L I N R D A Y L S M I Y R A Q S I S E I K T I R A D L E S L V K S F Q N E E 407
AGTGACTCTAAAGTAGAGCCTGAAAGTCCCGTTAAAGTAGAAAAACCAGTTGATGAAGAAAAACCTAAAGATCAAAAGAAGCTAGTTGATCAATCAAAACCCGAATCGAATTCAAAAGAA
S D S K V E P E S P V K V E K P V D E E K P K D Q K K L V D Q S K P E S N S K E 447
GGGTGGATTAAGAAAGATAATAAGTGGTTCTATATTGAGAAATCAGGTGGAATGGCAACAGGTTGGAAGAAGGTAGCAGACAAATGGTACTACCTCGATAATACGGGTGCTATAGTTACG
G W I K K D N K W F Y I E K S G G M A T G W K K V A D K W Y Y L D N T G A I V T 487
R1 R2
GGTTGGAAGAAGGTAGCAAACAAATGGTACTATCTTGAAAAATCAGGTGCGATGGCAACAGGATGGAAGAAAGTATCAAACAAGTGGTACTACCTTGAAAACTCAGGTGCAATGGCAACA
G W K K V A N K W Y Y L E K S G A M A T G W K K V S N K W Y Y L E N S G A M A T 527
R3 R4
GGATGGAAGAAAGTATCAAACAAGTGGTACTACCTTGAAAATTCAGGCGCAATGGCTACAGGATGGAAAAAGGTAGCAAACAAATGGTACTACCTTGAAAACTCAGGTGCGATGGCAACA
G W K K V S N K W Y Y L E N S G A M A T G W K K V A N K W Y Y L E N S G A M A T 567
R5 R6
GGATGGAAGAAAGTATCGAACAAGTGGTACTACCTTGAAAACTCAGGCGCAATGGCTACAGGATGGAAAAAGGTAGCAAACAAATGGTACTACCTTGATAAATCAGGAATGATGGTTACA
G W K K V S N K W Y Y L E N S G A M A T G W K K V A N K W Y Y L D K S G M M V T 607
R7 R8
GGTTCAAAATCTATTGATGGTAAAAAGTATGCATTTAAGAACGATGGAAGTTTAAAATAGACGGGATATCCCGTCTATTTTTTATGAATTTATGAAATTATCATTTGTATATATATTTAT
G S K S I D G K K Y A F K N D G S L K * 626
R9

FIGURE 2

```
Epa    GWIKKDNKWFYIEKSGGMAT 467
       **.. ...*.*.. .*.***
PspA   GWLQYNGSWYYLNANGAMAT 462

Epa    GWKKVADKWYYLDNTGAIVT 487
       ** **...****...**..*
PspA   GWAKVNGSWYYLNANGAMAT 482

Epa    GWKKVANKWYYLEKSGAMAT 507
       ** . ...****. .*****
PspA   GWLQYNGSWYYLNANGAMAT 502

Epa    GWKKVSNKWYYLENSGAMAT 527
       ** **...****...*****
PspA   GWAKVNGSWYYLNANGAMAT 522

Epa    GWKKVSNKWYYLENSGAMAT 547
       ** . ...****...*****
PspA   GWLQYNGSWYYLNANGAMAT 542

Epa    GWKKVANKWYYLENSGAMAT 567
       ** **...****...*****
PspA   GWAKVNGSWYYLNANGAMAT 562

Epa    GWKKVSNKWYYLENSGAM-A 586
       ** * ...*****.**** *
PspA   GWVKDGDTWYYLEASGAMKA 582

Epa    TGWKKVANKWYYLDKSGMMV 606
       . * **..****..  * .
PspA   SQWFKVSDKWYYVNGLGAL- 601

Epa    TGSKSIDGKKYAFKNDGSLK 626
       . ....** *
PspA   AVNTTVDGYKVNANGEWV   619
```

FIGURE 3

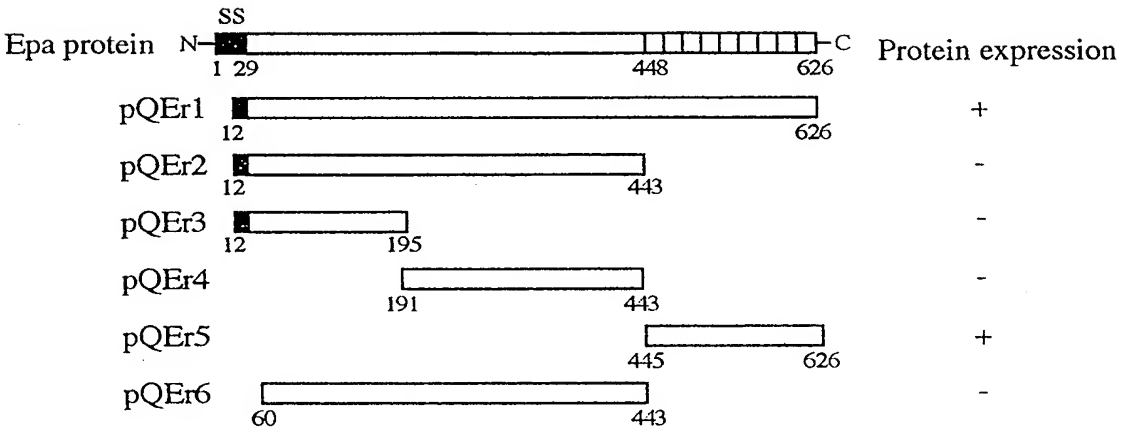
SS
Epa protein
N
C
1 29
448
626
Protein expression
pQEr1
12
626
+
pQEr2
12
443
-
pQEr3
12
195
-
pQEr4
191
443
-
pQEr5
445
626
+
pQEr6
60
443
-

FIGURE 4

INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/US 00/03789

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/195 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	IMADA YUMIKO ET AL: "Truncated surface protective antigen (SpaA) of Erysipelothrix rhusiopathiae serotype 1a elicits protection against challenge with serotypes 1a and 2b in pigs." INFECTION AND IMMUNITY, vol. 67, no. 9, September 1999 (1999-09), pages 4376-4382, XP000914965 ISSN: 0019-9567 the whole document --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2000	17/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter: nal Application No
PCT/US 00/03789

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SHIMOJI YOSHIHIRO ET AL: "Immunological characterization of a protective antigen of Erysipelothrix rhusiopathiae: Identification of the region responsible for protective immunity." INFECTION AND IMMUNITY, vol. 67, no. 4, April 1999 (1999-04), pages 1646-1651, XP000914977 ISSN: 0019-9567 the whole document	1-27
X	GALAN J E ET AL: "Cloning and expression in Escherichia coli of a protective antigen of Erysipelothri rhusiopathiae" INFECTION AND IMMUNITY, vol. 58, no. 9, 1990, pages 3116-3121, XP000915002 WASHINGTON US the whole document	1,7,19, 28
X	SOU-ICHI MAKINO ET AL: "Protperites of repeat domain found in a novel protective antigen, SpaA, of Erysipelothirx rhusiopathiae" MICROBIAL PATHOGENESIS, vol. 25, 1998, pages 101-109, XP000922807 figure 3; table 3	13-18

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DERWENT-ACC-NO: 2000-524541

DERWENT-WEEK: 200062

TITLE: Vaccines for protecting turkeys and pigs against Erysipelothrix rhusiopathiae infections comprising a polypeptide sequence from the N-terminal region of an erysipelas protective antigen

INVENTOR: FISCHETTI V A; SHIMOJI Y

PATENT-ASSIGNEE: UNIV ROCKEFELLER[UYRQ]

PRIORITY-DATA: 1999US-119389P (February 10, 1999)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE
WO 0047744 A1	August 17, 2000	EN
AU 200033640 A	August 29, 2000	EN

DESIGNATED-STATES: AE AL AM AT AU AZ BA BB BG BR BY CA CH CN CR CU CZ DE DK DM EE ES FI GB GD GE HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG U S UZ VN YU ZA ZW AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW NL OA PT SD SE SL SZ TZ UG ZW

APPLICATION-DATA:

PUB-NO	APPL-DESCRIPTOR	APPL-NO	APPL-DATE
WO2000047744A1	N/A	2000WO-US03789	February 10, 2000
AU 200033640A	Based on	2000AU-033640	February 10, 2000

INT-CL-CURRENT:

TYPE	IPC DATE
CIPS	C07K14/195 20060101
CIPS	C12N15/31 20060101

ABSTRACTED-PUB-NO: WO 0047744 A1

BASIC-ABSTRACT:

NOVELTY - Vaccines (II) containing immunogenic polypeptides (I) (or the nucleic acids (III) that encode them) which comprise immuno-protective groups from the N-terminal region of an erysipelas protective antigen (Epa) isolated from Erysipelothrix rhusiopathiae, are new.

DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:

(1) a polypeptide (I) comprising an amino acid sequence corresponding to 25 contiguous amino acids from (A1) (a defined 626 amino acid sequence (A1) given in the specification) from between amino acids 30 to 447;

(2) a vaccine (II) comprising an immunogenic polypeptide of Erysipelothrix rhusiopathiae (the immunogenic peptide comprises an immuno-protective group from an N-terminal region of an erysipelas protective antigen (Epa) and an adjuvant);

(3) a nucleic acid (III) encoding a polypeptide comprising an amino acid sequence corresponding to 25 contiguous amino acids of, or about 85% identical to, amino acids 30 to 447 of (A1);

(4) an expression vector (IV) comprising (III) (the nucleic acid is DNA);

(5) a method (V) for protecting an animal from infection by E. rhusiopathiae, comprising administering (I), (III) and/or an antibody generated against or purified with an immuno-protective group from an N-terminal region of an erysipelas protective antigen (Epa) to the animal;

(6) a method (VI) for detecting the presence of protective antibodies to E. rhusiopathiae, comprising detecting antibodies from a sample with a polypeptide comprising an immuno-protective group from the Epa protein of E. rhusiopathiae under conditions suitable for binding of the antibody to the polypeptide; and

(7) a kit (VII) for detecting the presence of antibodies to E. rhusiopathiae, comprising a polypeptide containing the immuno-protective group and an antibody detector.

Vaccine.

N-terminal region coding sequences were cloned into plasmid vectors for expression in Escherichia coli. The E. coli clones were grown at 28 degreesC and a clone containing plasmid pQEr3 (containing a coding sequence for amino acid residues 12-195 of Epa) produced a small amount of recombinant protein. The protein was purified and used to immunize mice.

Mice were challenged sub-cutaneously with 70 LD50 cells of Fujisawa-SmR and observed for clinical symptoms and death for 14 days. All 5 mice immunized with the 12-195 fragment of Epa protein survived without any clinical symptoms. However, all 5 control mice died 5 days after challenge. The arithmetic mean of ELISA (enzyme linked immunosorbant assay) immunoglobulin (Ig)-G titers taken before bacterial challenge from individual sera of vaccinated mice was 76800 against peptide 12-195 (using an optical density end point of 1.0).

USE - The vaccine compositions (II), polypeptides (I) and (III) may be used to immunize pigs and turkeys against infection by Erysipelothrix rhusiopathiae (claimed), the major causative agent of erysipelas in animals and erysipeloid in humans. In pigs the organism causes acute septicemic disease or chronic disease characterized by endocarditis and polyarthritis. The polypeptides may also be used to assay for the presence antibodies against this organism in biological samples (claimed).

EQUIVALENT-ABSTRACTS:

BIOTECHNOLOGY

Preferred Proteins: (I) comprises a sequence corresponding to 25 contiguous amino acids from between amino acids 30 to 195 of (A1) (a defined 626 amino acid sequence given in the specification). Preferably (I) comprises (A2):

SLMSCLLLTAMPLQTAFADSTDISVIPLIGEQVGLLPVLPGTGVHAQEYNKMTDAYIEKLVSLINQKVKPFLI
EPKGYQSFEAVNEEINSIVSELKNEGMSLQNIHHMFKQSIQNLATRIGYRSFMQDAMYLENFERLTIPELDEA
VDLLVNYEVKHRILVKYEGKVKGRAPLEAFIVPLRD (A2)

(I) is a fusion protein comprising the immuno-protective epitope and a purification handle or a carrier polypeptide.

Preferred Vaccines: The immuno-protective group is a 25 amino acid segment located in the N-terminal of Epa as depicted between amino acids 30 to 447 of a defined 626 amino acid sequence (A1) given in the specification. Preferably, it is derived from between amino acid residues 30 to 195, especially residues 30 to 100 of (A1). Preferably, the immunogenic peptide may comprise (A2).

The immunogenic polypeptide may be a fusion protein comprising the immuno-protective epitope and a purification handle or a carrier polypeptide.

Preferred Nucleic Acids: (III) encodes a fusion protein comprising the immuno-protective epitope and a purification handle or a carrier polypeptide.

Preferred Vectors: (III) is a viral or non-viral vector.

Preferred Methods: (V) is used to treat a turkey or pig. The immuno-protective group is a 25 amino acid fragment located in the N-terminus of Epa, comprising a region from between amino acids 30 to 447, especially amino acids 30 to 195 of (A1).

Preparation: The nucleic acids, polypeptides and vaccine compositions may be produced according to standard methodologies.

SPECIFIC POLYPEPTIDES

(I) comprises a sequence corresponding to 25 contiguous amino acids from between amino acids 30 to 195 of (A1) (a defined 626 amino acid sequence given in the specification). Preferably (I) comprises (A2):

SLMSCLLLTAMPLQTAFADSTDISVIPLIGEQVGLLPVLPGTGVHAQEYNKMTDAYIEKLVSLINQKVKPFLI
EPKGYQSFEAVNEEINSIVSELKNEGMSLQNIHHMFKQSIQNLATRIGYRSFMQDAMYLENFERLTIPELDEA
VDLLVNYEVKHRILVKYEGKVKGRAPLEAFIVPLRD (A2)

TITLE-TERMS: VACCINE PROTECT TURKEY PIG ERYSIPELOTHRIX INFECT COMPRISE POLYPEPTIDE SEQUENCE N TERMINAL REGION ERYSIPELAS ANTIGEN

DERWENT-CLASS: B04 C06 D16

CPI-CODES: B04-B04C1; B04-B04M; B04-C01G; B04-E03F; B04-E08; B04-F0100E; B04-G07; B04-N03A0E; B04-P01; B11-A; B11-C07A4; B11-C08E1; B11-C09; B12-K04E; B12-M07; B14-A01B; B14-S11B; C04-B04C1; C04-B04M; C04-C01G; C04-E03F; C04-E08; C04-F0100E; C04-G07; C04-N03A0E; C04-P01; C11-A; C11-C07A4; C11-C08E1; C11-C09; C12-K04E; C12-M07; C14-A01B; C14-S11B; D05-A01A4; D05-A01B; D05-C12; D05-H04; D05-H07; D05-H08; D05-H09; D05-H11; D05-H12A; D05-H12E; D05-H14; D05-H17A5; D05-H18;

CHEMICAL-CODES: Chemical Indexing M1 *01* Fragmentation Code M421 M423 M710 N135 N161 P001 P220 Q233 Specific Compounds RA00NS Registry Numbers 93605

Chemical Indexing M1 *02* Fragmentation Code M421 M423 M710 M781 N102 N135 N161 P001 P220 P831 Q233 Q505 Specific Compounds RA00H3 Registry Numbers 184616

Chemical Indexing M1 *03* Fragmentation Code M421 M423 M750 N102 N135 N161 P001 P220 P831 Q233 Specific Compounds RA00C8 Registry Numbers 184587

Chemical Indexing M6 *04* Fragmentation Code P001 P220 P831 Q233 Q505 R515 R521 R627 R630

SECONDARY-ACC-NO:

CPI Secondary Accession Numbers: 2000-155849